# GENOTOXIC STUDIES IN *ETROPLUS SURATENSIS* (BLOCH) USING MICRONUCLEUS TEST

DISSERTATION SUBMITTED BY

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# C E R T I F I C A T E

This is to certify that this Dissertation is a bonafide record of work carried out by Shri. M. Muruganandam under my supervision and that no part thereof has been presented before for the award of any other Degree.

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## ABBREVIATIONS USED

| | | |
|---|---|---|
| MN | - | Micronucleus. |
| NA | - | Nuclear Anomaly |
| NL | - | Nuclear Lesion |
| SCE | - | Sister Chromatid Exchange |
| CA | - | Chromosome Aberration |
| Cd | - | Cadmium |
| CP | - | Cyclophosphamide |
| MMC | - | Mitomycin-C |
| CD | - | Critical Difference |

# PREFACE

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The unlawful introduction of potential pollutants to the aquatic environment and the apparently increasing quantum of diversified synthetic and natural chemicals having adverse effects on the biota have proved to be a risk factor to human resources. The science of aquatic toxicology has already revealed great dangers from the deliberate dumping of potential pollutants in aquatic environment and emphasizes the need to investigate the extent to which the genotoxic substances in the aquatic environment present a risk to aquatic organisms and man and the need for further regulation.

Though a vast amount of information has been generated in the country on different aspects of aquatic pollution, the concepts embodied in 'genetic toxicology', 'environmental mutagensis' and 'genotoxicity' of chemicals or environmental genetic damage have not been given due recognition in pollution studies.

Genetic toxicology is a new field of multidisciplinary science with different realistic probes for environmental and chemical screening. Genotoxicity assays identify and analyse the action of agents with toxic effects directed towards the heriditary components of living system.

For the aquatic environment, aquatic organisms and their associated higher trophic organisms including man, the genetic monitoring using different genetic biomarkers in different model systems including aquatic vertebrates and invertebrates, has proved to be an effective approach in recent years all over the world. Out of the many genetic monitors (biomarkers), the short-term assay, 'Micronucleus Test' is opined to be a rapid and facile technique of screening for genotoxicity.

Owing to the direct link with human food chain, greater susceptibility to environmental toxicants, and moderate similarity in metabolic systems with human beings, fish have proved to be one of the best model system for genotoxicity evaluation.

Etroplus suratensis due to its suitability for toxic studies, economic importance, and local abundance has been chosen as a test species for the present study. Three known pharmaceutical mutagens, (Colchicine, Cyclophosphamide (CP), Mitomycin-C (MMC)) and one heavy metal (cadmium) whose mutagenic potentials have been well documented were selected to test the response of the test species at the level of Micronuclear manifestations.

The main objectives of the present study were (i) to detect various types of nuclear anomalies of fish erythrocytes, (ii) to detect artifacts seen in piscine erythrocytic micronucleus test (iii) to generate base-line data to screen known chemicals for genotoxic potential and (iv) to assess genotoxic effects in fish from suspected polluted sites with a standardised protocol for piscine Micronucleus Test.

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# INTRODUCTION

# I N T R O D U C T I O N

The consequences of environmental pollution in the form of Genotoxic and Cytotoxic effects on fish and shell-fish have created an interest among toxicologists and patholigists since the last centuary mainly because the consequences were similar to conditions occurring in mammals, including man. The origin, effects and detection of genotoxicity in aquatic species have been the subject of several research and review papers (Berry, 1980; Beardmore et al., 1980; Kocan et al., 1982; Metcalfe, 1988; Capuzzo et al., 1988).

Pollution means the introduction by man, directly or indirectly, of substances or energy into the aquatic environment resulting in such deleterious effects as harm to living resource, hazards to human health, hindrance to aquatic activities including fishing impairment of quality for use of water and reduction of amenities (GESAMP, 1991). The different components of Biological, physical and chemical contaminants which contribute to the global contamination are shown in fig. 1.

Further, the number of synthetic chemicals in commercial use today amounts to about 1,00,000 showing an annual rise of 1000 chemicals over the years. These chemicals often have

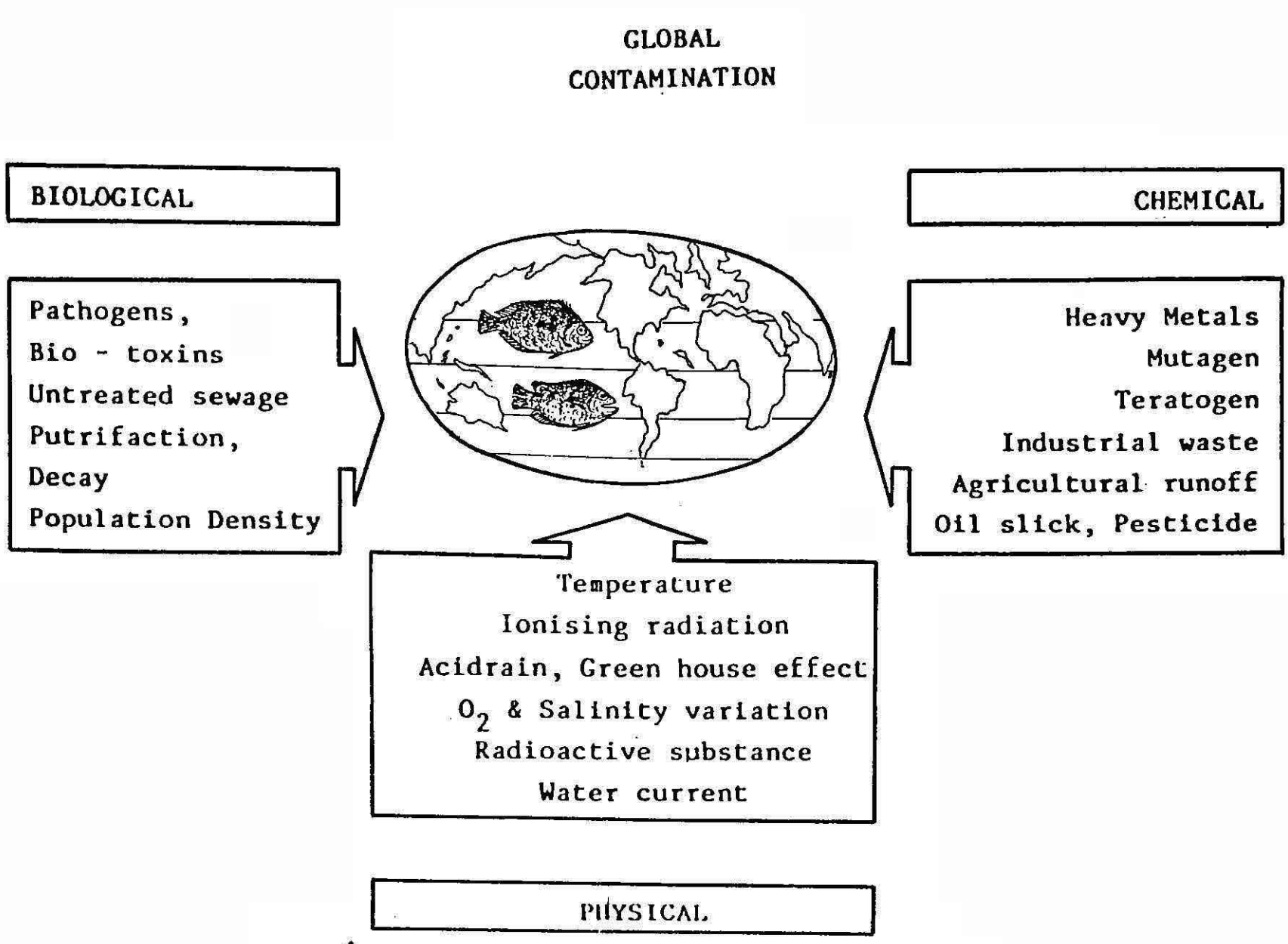



Fig. 1. COMPONENTS OF GLOBAL CONTAMINATION

( Sindermann, 1980 )

adverse side effects on biota and demands the need for hazard evaluation using animal models to extrapolate to human system (Protic and Aleksander-Sabljic, 1989).

Genotoxicants, carcinogens and teratogens are often encountered in food additives, pharmaceuticals, pesticides, cosmetics, industrial, house-hold and municipal wastes. No environment is free from mutagens (Manna, 1982) and all living organisms including man are exposed to toxic and hazardous chemicals which act genetically either directly through mutations (Parry et al., 1976) or by exerting selection pressure (Beardmore et al., 1980). Fish in their natural environments are also subjected to numerous stressors including genotoxic agents which cause long-term genetic impairment (Pesch and Pesch, 1980).

Many chemicals are genotoxic and cause mutagenesis in animals. The genotoxic mechanism is one where the genotoxicant or one of its metabolites reacts with DNA. Because of the Universality of the DNA molecule an agent which is genotoxic for one group of living organisms is typically genotoxic for other groups (Landolt and Kocan, 1983).

Mutagenic compounds may lead to changes in the gene pool with unpredictable consequences on population genetics (Alink et al., 1980) and hence it would not be out of place to surmise the decline or drastic fluctuation in fisheries due to

such compounds (Dass, 1990). There are two possible causes of immediate concern the first is the direct impact of genotoxins on aquatic organisms, the second is indirect impact on man via exposure to genotoxins through aquatic environment. Since the levels of all such substances in the aquatic environment are very low, direct exposure of man is assumed to be insignificant and indirect exposure, via aquatic organisms consumed as food, is the main exposure pathway that needs to be stressed upon.

The main hazard caused by a mutagen is bioaccumulation in the food species which may be ingested by man. The mutagen ingested through the contaminated food reach and accumulate in the blood and organs. The bio-accumulated mass of the contaminants at lethal level leads to death of the individual whereas at sublethal level it leads to immunotoxicity, genotoxicity, carcinogenicity, teratogenicity, etc.,(Fig. 2). It is pertinant to record that animal products like fish meat and other sea foods are vital components of our food chain which can transfer toxic chemicals to sea food consumers. Examples of such transmission of mutagens through food to man are mercury from fish in contaminated Swedish lakes and a toxic compound in bracken (a large coarse fern) which was detected in milk of cows feeding on marginal hill pasture (Berry, 1980). The grimness of the Minamata tragedy caused by the consumption of mercury contaminated fish and shellfish and the

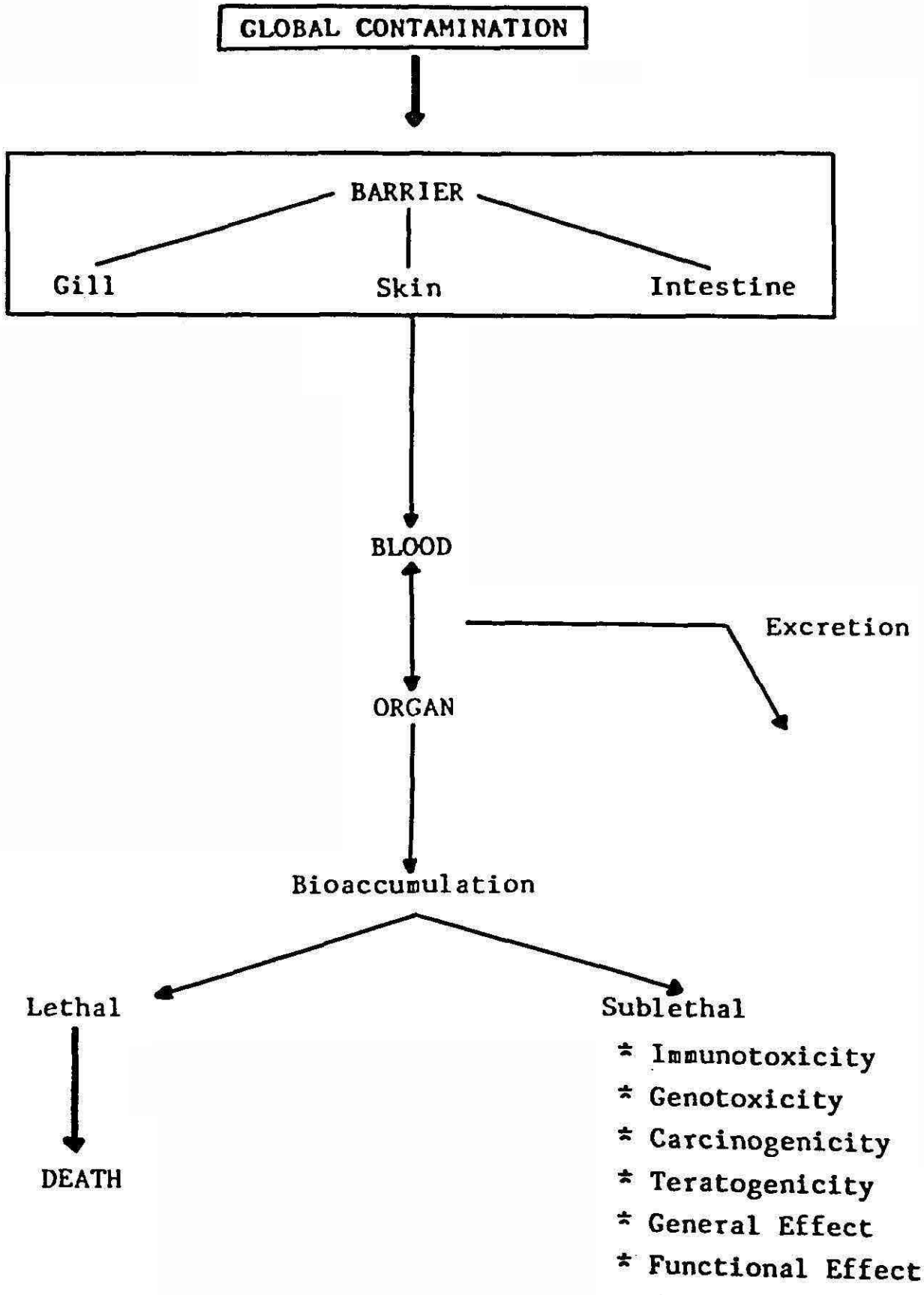



Fig:- 2. BIOACCUMULATION AND TOXIC EFFECTS OF POLLUTANTS

( Boudou et al., 1983 )

out break of "Itai itai" caused by the consumption of food contaminated with cadmium and the ill effects of atmospheric lead, are few of the important mishaps.

From a human health stand point, at normal levels of consumption and contamination, the data on consumption of potential genotoxins/carcinogens via marine fish and shellfish give little reason for concern in relation to most of the substances considered. However, there may be increased risks if fishery products are abnormally contaminated with genotoxins. Therefore, there is a need for continuous vigilance and control over the disposal of known genotoxins into the environment. Extensive research is necessary to establish cause and effect relationships between genotoxins and aquatic organisms.

In fish, genetical changes could be a useful indicator of the effects of pollution and provide a quantitative and qualitative measure of the biological damage being produced by the commercial chemicals/pollutants and hence genetic bio-markers for environmental monitoring and chemical hazard evaluation are now being recognised by aquatic toxicologic researchers.

Genetic toxicity assay has been enriched by the large number of methods for characterising the genotoxicity of chemicals and other environmental pollutants. The classical method of assessing genotoxic effects is the examination of

metaphase chromosome preparations of cells exposed to test agents or environmental pollutants either in vitro or in vivo. Unfortunately, this approach is tiresomely long and often has low precision. Unlike mammals, many species of fish have extremely small and numerous irregular choromsomes which present severe difficulty in resolution, observation and characterization of chromosomal defects such as deletions, alterations and sister chromatid exchanges (SCE). Micronucleus (MN) test is exempted from all the problems encountered in the metaphase chromosomal preparations and hence considered to be an inexpensive test.

The micronucleus is small membrane bound mass of chromatin material present in the cytoplasm of dividing cell populations resulting from chromosome aberrations (CA) in the cell cycle, following clastogenic exposure (Heddle et al., 1983), either occurring fused with the Principal nucleus or remaining as a seperate, small secondary nucleus in the cells, scorable in telophase stage. In no case micronucleus and chromosomal damage are shown to occur independently of one another in a dividing cell population (Heddle et al., 1983) and hence scoring of micronuclei may indicate chromosome aberrations and genotoxic effects. Owing to the inherent advantages of piscine erythrocytic micronucleus assay over micronucleus assays involving different tissues in different organisms, it is presumed to be an inexpensive, reliable, and rapid screening test. The greater reliability and sensitivity of the micronucleus test is substantiated

by Ames test and SCE (Landolt and Kocan, 1983). Its presence represents both clastogenic and spindle apparatus damage. Although the piscine micronucleus assay is an excellent screening test, information on the suitability of the test in environmental screening and chemical hazard evaluation is scanty. The present work was taken up to bridge gaps between the records and lacunae in such a way so as to standardise the piscine micronucleus test and to evaluate its suitability for environmental screening and chemical hazard analysis. The known chemicals, viz. colchicine, cyclophosphamide, mitomycin-C and cadmium were taken as genotoxicants to study their genotoxic effect in E. suratensis.

Colchicine is metablized to a mixture of compounds and are known to produce genotoxic effects in higher vertebrates (Flower et al., 1980). Majone et al.(1990) studied the genotoxicity of colchicine in mussel (Mytilus galloprovincialis).

Cyclophosphamide (CP) as such is not active, but is activated by P-450 mixed function oxidases to the toxic state (Mohn and Ellenberger, 1976). The studies carried out by Kligerman (1979 c), Kligerman et al. (1981), Krishnaja and Rege (1982) proved that cyclophosphamide is genotoxic to fishes. Mitomycin-C (MMC) is enzymatically activated to function as a bifunctional alkylating agent, preferentially at $O_6$ of guanosine (Rang and Dale, 1987). Kocan et al. (1982) studied the genotoxic effects of mitomycin-C and

cyclophosphamide as cytotoxic agents to fish. Mitomycin-C prevented cell division and subsequently reduce the frequency of micronucleus (Das and Nanda, 1986). Majone et al. (1987) have studied the genotoxicity of mytomycin-C in a marine mussel.

Cadmium is an environmental poison that accumulates and cause damage to Kidney and blood cells both in mammals (Larsson, 1975; Nomiyama et al., 1975; Klaassen, 1980) and in aquatic organisms (Gardner and Yevich, 1970). Gardner and Yevich (1970) studied the nuclear anomalies similar to mammals produced by cadmium in Fundulus heteroclitus. Intraperitoneal injection in low quantity of cadmium to Pleuronectus platessa caused strong reduction in Cytochrome P-450 dependent ethoxyresorufin o-deethylase (EROD) activity and no significant alteration in the activity of phase II enzyme, glutathione-s-transferase even after six days of exposure (Johnsson-Sjobeck and Larsson, 1978). Gill and Pant (1985) observed different nuclear anomalies after cadmium exposure to Puntius conchonius.

All the DNA damage, chromosomal aberrations and interventions in cell cycle depend on chemical and physical properties of the xenobiotics and their metabolites, species, age, sex, dose and routes of exposure, cell metabolic state and various environmental factors (Buhler and Williams, 1988) and they can be manifested through micronucleus (Estmond and Tucker, 1989).

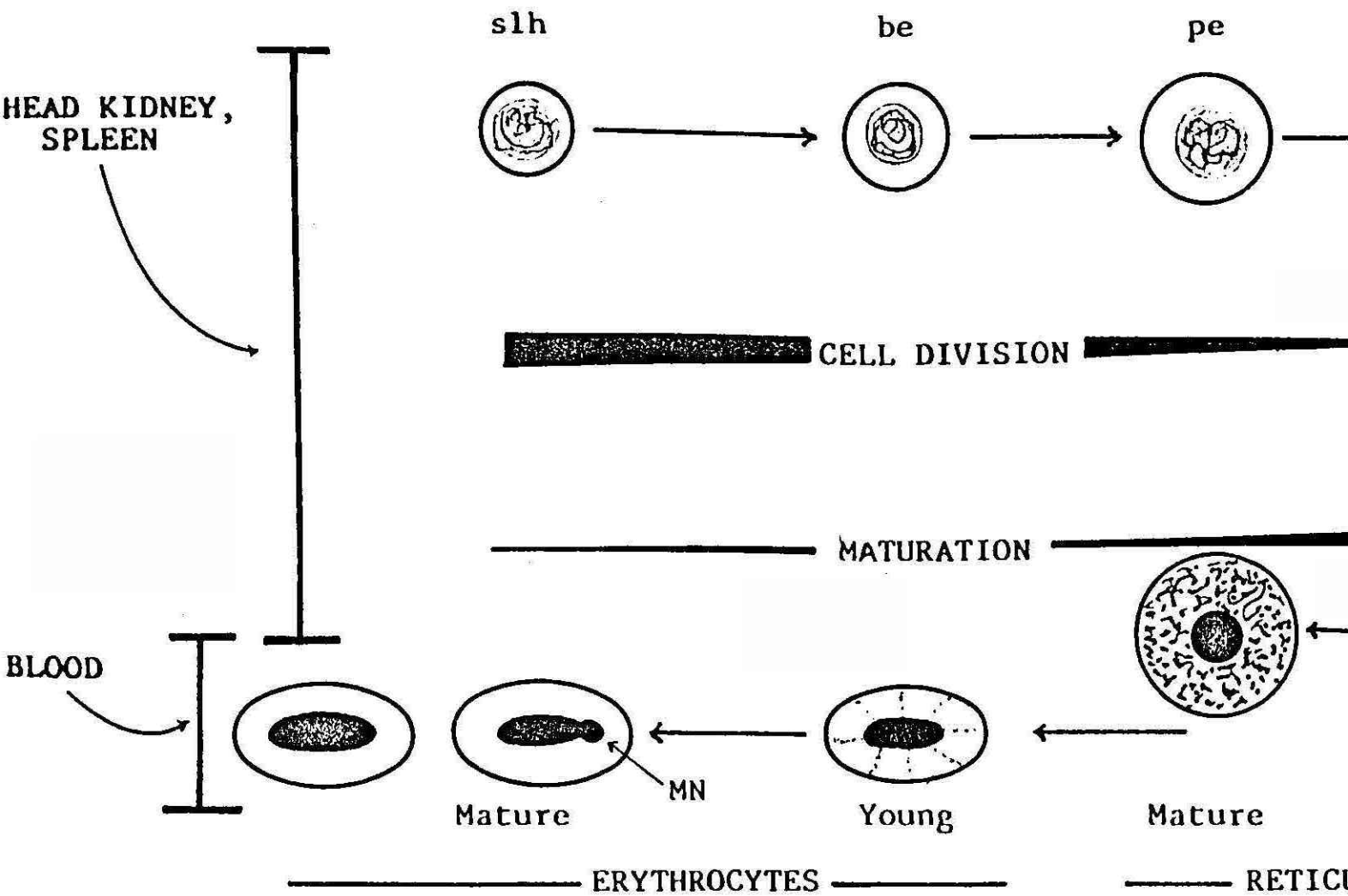
Due to the impairment of the spindle structure, the anaphase laggards in erythroblasts undergoing last chromosome replication and mitosis are set aside from the main nucleus and form micronucleus (Fig. 3).

Genetic damage exerted by colchicine, cyclophosphamide, mitomycin-C and cadmium were studied through micronucleus induction in mammals by a number of investigators including Evans et al. (1959), Salamone et al. (1980) and Heddle et al. (1983). But the records of micronucleus induction by chemicals on fish are very few (Hoeven et al., 1982; Das and Nanda, 1986; Manna and Sadhukhan, 1986) .

## Toxicology

Toxicology is a branch of medical science, which deals with the source, characters and properties of poisons, the signs, symptoms, diagnosis and treatment of poisoning and physiological and chemical tests by means of which poisons can be detected (Teacher, 1970).

Environmental toxicology deals with the incidental exposure of tissue to chemicals that are basically contaminants of the environment, food or water (Hamilton, 1976).




slh, Small Lymphoid Haemoblast; be, Basophilic Er
pe, Polychromatic Erythroblast; ae, Acidophilic Erythroblas

Fig:-3. ERYTHROCYTE PRODUCTION AND MICRONUCLEUS FORMATION (M

Toxicology is categorised into two groups: the traditional toxicology and the modern toxicology. The former deals with testing of the animal for the detection of qualitative and quantitative changes in behaviour, homeostatic processes and lethality whereas the latter deciphers molecular events underlying the expression of toxic end points, and includes Genetic toxicology, Immunotoxicology, Teratology, Oncology, Pharmacology and Behavioural toxicology (Brusick, 1980).

Aquatic toxicology is stepping into molecular toxicology and is being enriched by a variety of techniques and approaches becuase of its utmost importance (Lloyd, 1980). Recent Public awareness of increasing contamination of the aquatic ecosystem, and the associated health risks inspired traditional toxicologists to utilise realistic probes to discern toxic effects at the cellular level and its prevention (Baksi and Frazier, 1990; Thomas, 1990; Marshall, 1993).

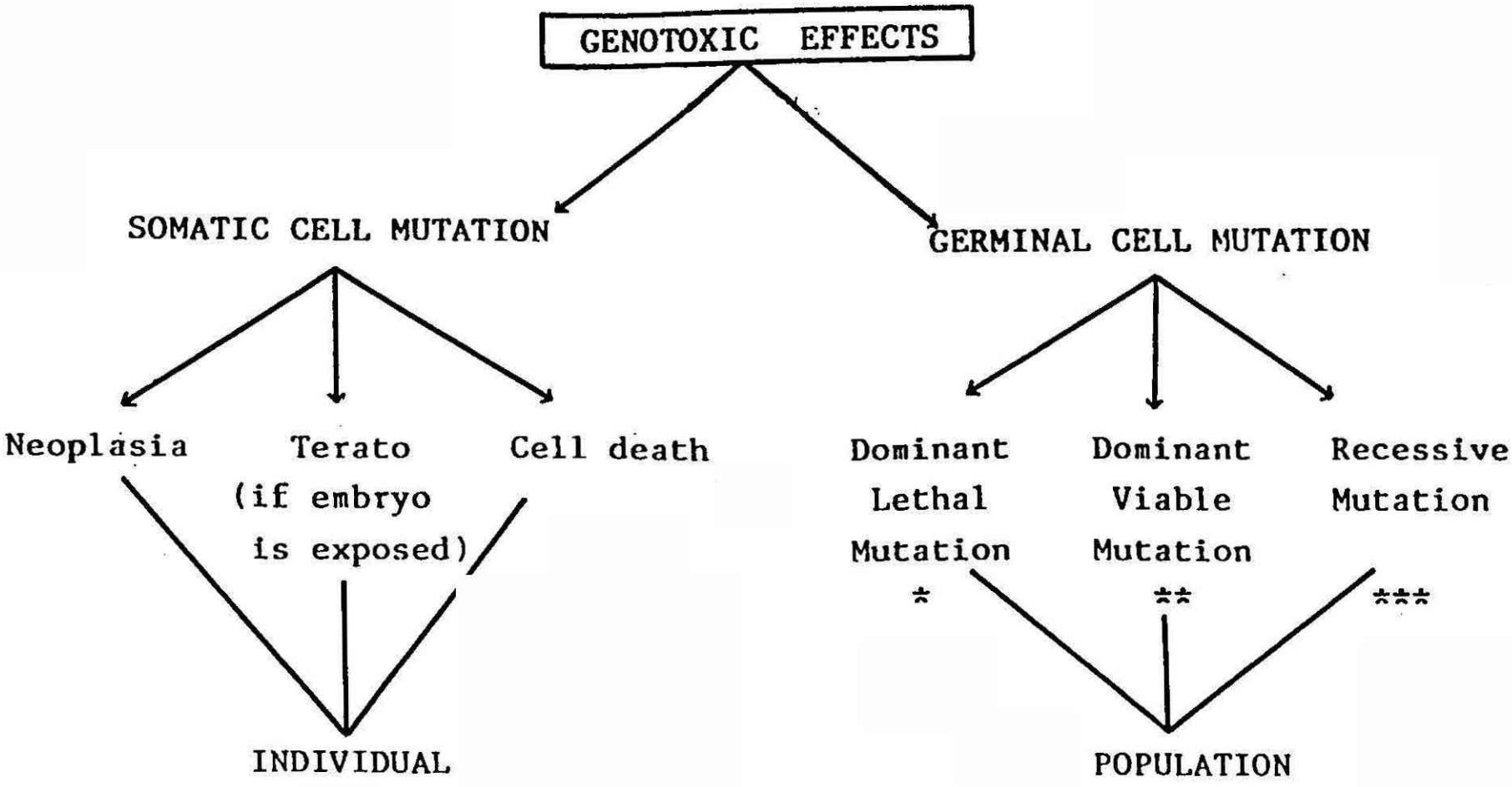
## Genetic Toxicology

Mutagenicity studies perhaps started with the formulation of mutation theory by de Vries (1901-1903) and the studies of mutagenic effects of radiation and chemicals by Muller (1927) and Auerbach & Robson (1946) respectively. The exploratory studies started only in the sixties using several mutagenicity testing methodologies in mammals (Evans, 1959), aquatic organisms (Regan et al., 1968) and other models.

Pioneering studies were on dominant lethal test in mice (Rohrborn, 1970) somatic and germinal cells (Brookes and Lawley, 1971),transformation in microorganisms (Ames, 1971), induced mutation in yeast (Mortimer and Manney, 1971), genotoxic observation in fish (Tystsugima,1972), micronucleus test (Heddle, 1973) and sister chromatid exchange (SCE) analysis (Perry and Wolf, 1974, Latt, 1974a).

Genotoxicity studies identify and analyse the action of toxicants directed towards the hereditary components of living systems. Agents specifically producing genetic alterations at sub-toxic exposure levels which result in organisms with altered hereditary characteristics are called genotoxic (Brusick, 1980). Consequences of genotoxic effect at individual and population level, has been shown in fig.4. Genotoxic effects of chemicals can be classified into micro and macrolesions as described in fig.5.

Genetic toxicology studies have orginated from mammalian species (Brusick, 1980) and has extended to aquatic organisms (Beardmore et al. 1980) by rapid evolution to diversified genetic models including plants. Genetic toxicology testing has been enriched by the large number and diverse sets of end points for characterising the genotoxicity of agents, viz. chromosome aberration (CA), micronucleus test, sister chromatid exchange, dominant lethal mutation, heritable translocations, unscheduled DNA synthesis and other tests to detect gene mutations using microbes and cell cultures (Daniel and Golberg, 1980).




(* Contribution to genetic burden )

Fig. 4 CONSEQUENCES OF GENOTOXIC EFFECTS (Bruscik, 1980)

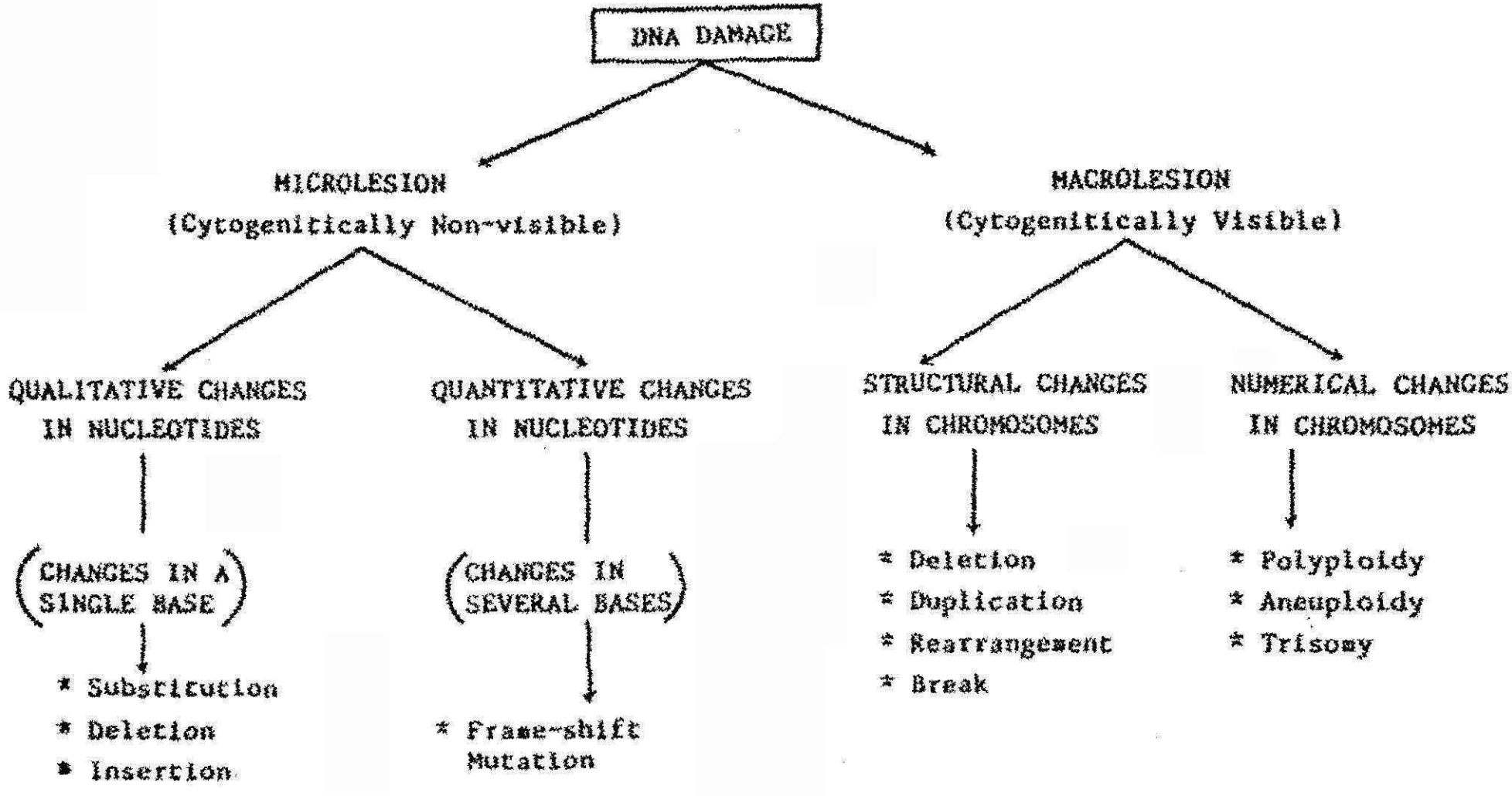



Fig:- 5 CLASSIFICATION OF GENOTOXIC EFFECTS (Brusick, 1980)

Berry (1980), Beardmore et al. (1980) and EPA (1984) reviewed the various genetic aspects of pollution monitoring and opined the need to study the distribution of genotoxic substances and their effects on gene pools. Following this, Manna (1986, 1989) reviewed genotoxic studies in fish and opined, tilapia to be a good cytogenetic model for testing genotoxicity. Subsequently Thomas (1990), Mitrofanov et al. (1991), Pechkurenkov (1991), Liu et al. (1991), Nishimoto et al. (1991),Zhang et al.(1992), Bailey et al . (1992), Yasuhira et al. (1992), Reichert et al. (1992), Schnitz and O' Connor (1992), Kubota et al. (1992), Adams et al. (1992)& Aoki et al. (1993) have studied various aspects of fish genotoxicity using different species including, various freshwater carps, rainbow trout, gold fish, tilapia, medaka, eastern mudminnow, etc.

In genetic toxicology different end points varying in methodology and sensitivity are used for environmental and chemical screening, viz. SCE, chromosome aberration, micronucleus test.

Sister Chromatid Exchange (SCE)

Theoretically SCE represents an visual manifestation of a reciprocal exchange of identical DNA material between two sister chromatids.

SCE Analysis using a DNA analong bromodeoxy uridine (BrdU) has been proposed as a new cytogenetic method for determining the potential hazards of chemicals in the

environment(Latt, 1974 a, 1974 b; Perry and Evans, 1975; Kato and Shimada, 1975). SCE analysis in fish was employed by Kligerman and Bloom (1976), Barker and Rackham (1979), Alink et al. (1980), Stromberg (1981), Kerkhoff and Gagg (1985), Harrison (1987), Wei (1987) and Wrisberg and Von der Gaag (1992).

SCE assay and the micronucleus assay, are standard in vivo test systems for determining genotoxic responses in animals. However, the SCE assay and other metaphase assays are not suitable genotoxicity tests for most teleost species (Williams and Metcalfe, 1992).

Chromosome aberration

Chromosome aberrations can arise out of changes in chromosome number (gain or loss of single chromosome or set of chromosome) or changes in chromosome structure (breaks, deletions, rearrangements, etc.,fig. 5).

In mammals extensive work has been carried out since last three decades. Among aquatic organisms, fish have received considerable attention. Regan et al. (1968) reported chromosome aberration in marine fish. Consequently Tystsugima (1972), Kligerman et al. (1975), Sugatt (1978), Hooftman (1981), Krishnaja and Rege (1982), Yamazaki (1983), Al-Sabti (1985 a, b), Goodier et al. (1987), Walton et al. (1988) have

reported the incidence of chromosome aberrations in aquatic organisms. Due to the inherent inabilities and difficulties, the chromosome aberrations as a genotoxic end point has limited use over micronucleus test (Metcalfe, 1988; Williams and Metcalfe, 1992).

Micronucleus Test

Mirconuclei are the chromatin materials not included in the main nucleus presumably because of their failure to segregate to the progeny nuclei, a resultant of cell division, for different reasons have been lagging in anaphase, causing either chromosome breakage (acentric micronuclei) or malfunction of spindle apparatus (centric micronuclei) scorable in telephase stage. Micronucleus test yields information similar to that observed from Ames test (Jenssen and Ramel, 1980) and from conventional metaphase analysis (Kirkhart, 1981). Heddle et al. (1983) and others have listed out the basic advantages of micronucleus test, viz.

1. Scoring for micronuclei is considerably more than 10 fold faster than metaphase scoring. The test is time and cost effective. It is independent of a favourable karyotype. The end point score is easily recognised even by/with little formal training in cytogenetics.

2. Micronuclei can be detected in interphase even if cellular proliferation is required. The induced micronucleus persists at least through the next interphase and hence can be observed easily with no critical urgency.

3. The test can be applied to detect wide range of mutagens (Majone et al., 1988, 1990; Brunetti et al., 1992).

4. It is applicable both in laboratory and wild screening.

5. Reproducible results within and among laboratories can be achieved.

6. The test is a direct indication and realisation of genotoxicity. Higher micronucleus frequency indicates the greater genotoxicity.

7. The weakness and limitations of the test system can be factored into the data interpretation (Hayashi et al., 1984).

8. Good statistical power and amenability to substantial analysis because of more number of scorable cells (Hose et al., 1987).

9. Principally uniform protocols and evaluation criteria.

10. Multiple sampling is possible and hence chronological effects of an agents can be measured.

However, the micronucleus test is considered to be a less sensitive than Metaphase analysis. A serious limitation is that it cannot explain whether a given micronucleus is the end product of chromosomal break or a

whole chromosome lagged behind as a consequence of disturbance in spindle functioning (Hose et al., 1987; Carrasco et al., 1990).

Micronuclei are also called as Howell -Jolly bodies after Howell (1821) and Jolly (1905) who first discovered and described them in Red blood cells. Although the existence of micronucleus associated with chromosomal damage has been recognised by earlier workers especially in radiation field, the first serious attempt was made by Evans et al. (1959) and subsequently by Schroeder (1966, 1970) to use micronucleus as a monitor of cytogenetic damage. Schmid et al. (1971) and Heddle(1973) initiated studies to determine the parameters which could be used as useful indicators of cytogenetic damage in organisms. The work of Von Ledebur(1973) and Schmid (1975) led to the development of a simple in vivo test based on the identification of micronucleus in the Polychromatic erythrocytes of mouse bone marrow. The mammalian micronucleus test is a well known cytogenetic technique which assess genotoxic damage by the visual examination of cells for chromosome fragments.

The technique was first modified from bone marrow test to suit the peripheral blood cells in mammals by Mac Gregor et al.(198). Later, Hooftman and de Raat (1982) modified it for its application in fish. The modified technique was named "Piscine Micronucleus test" and was used in fish by several workers (Puffer et al., 1983; Manna et al., 1985; Smith et al., 1985; Das and Nanda, 1986;

Al-Sabti, 1986a; Hose et al., 1987; Mingde et al., 1987; Metcalfe, 1988; Carrasco et al., 1990; Daniels et al., 1992; Meier et al., 1992) for genotoxic evaluation.

Micronucleus tests, which utilise fish erythrocytes was termed as "Piscine erythrocytic micronucleus test".

In vivo Piscine erythrocytic micronucleus test is considered to be a better assay over mammalian in vivo assay because the activated metabolites reach target organs (bone marrow) to a lesser extent in mammals than in fish, where the target organ is kidney (Hose et al., 1987). In addition to this, application in vitro micronucleus test in aquatic organisms provides additional information (Gabriele et al., 1992).

In fishes, erythrocytic micronucleus test is most preferred than any other micronucleus test, since the erythropoietic cells, have high mitotic rate and erythrocyte production rate. Cells with high mitotic turnover is considered best for effective screening using micronucleus test (Metcalfe, 1988). Moreover, red blood cells can be obtained easily from fish blood without sacrificing the animal, which makes Piscine erythrocytic micronucleus test a biomonitor of the aquatic pollution (Hose et al., 1987). A brief account of the applications of Piscine erythrocytic micronucleus test by different workers has been given in Table-1.

Table 1 Genotoxic Chemicals/Agents/Effluents analysed in different fishes using Piscine Erythrocytic Micronucleus Test

| Sl.No. | Species | Genotoxic Agent/Chemicals/ Effluents | Reference |
|---|---|---|---|
| 1. | Umbra pygmaea (Eastern mudminnow) | Ethylmethane Sulphonate | Hooftman and de Raat (1982) |
| 2. | Oreochromis mossambica (Tilapia) | Aldrin, Cadmium Chlordie, D-Glucosamine, X-rays | Manna et al. (1985) |
| 3. | Cyprinus carpio (Carp) | Aflatoxin-B, Aroclor, Benzindine, Benzo(a) pyrene, Methyl Chloranthrene. | Al-Sabti (1986) |
| 4. | Heteropneustes fossilis | Mitomycin-C and paper mill effluent. | Das and Nanda(1986) |
| 5. | Pleurodeles walt (Newt) | Benzo (a) pyrene | Grinfield et al.(1986) |
| 6. | Monoptenes albus (Eel) | Chlorothalonil | Mingde et al.(1987) |
| 7. | Genyonemus lineatus (White croaker) | Environmental pollutants (Chlorinated hydrocarbons - DDTs and PCBs) | Hose et al.(1987) |

Table 1 contd..

| | | | |
|---|---|---|---|
| 8. | *Paralabrax clathratus* (Kelp bass) | Environmental pollutants (Poly cyclic aromatic hydrocarbons and Metabolites) | Hose *et al.*(1987) |
| 9. | *Umbra limi* (Mudminnow) | Ethyl methane sulphonate, Benzo (a) pyrene | Metcalfe (1988) |
| 10. | *Ictalurus nebulosus* (Bull head) | -do- | -do- |
| 11. | *Etroplus suratensis* (Pearlspot) | Aquatic pollutant (oil pollutants), Methylmethane Sulphonate, Cyclophosphamide, Methylparathion, Phosphamidon. | Dass (1990) |
| 12. | *Etroplus maculatus* (Orange chromide) | -do- | -do- |
| 13. | *Genyonemus lineatus* (White croaker) | Fluorescent aromatic compound, (FACs) | Carrasco *et al.*(1990) |
| 14. | *Oryzias latipes* (Medaka) | Coke oven waste water effluent with Bacterial load. | Meier *et al.*(1992) |

Besides the fish species, other aquatic organisms including invertebrates and amphibians were assessed for micronucleus test. Effectiveness of Mussel micronucleus test was studied by Gola et al. 1986, Brunetti et al. (1988, 1991, 1992), Majone et al. (1987, 1988, 1990), Gabriele et al. (1992), Wrisberg and Van der Gaag, (1992) to assess the genotoxic agents/effects of physical (temperature, etc.), chemical (Mitomycin-C, Colchicine, heavy metals including Cadmium) and biolgical responses (age, sex, etc.,). Similarly micronucleus test using amphibian species were analysed by Jaylet et al. (1986, 1990) and Von Hummelen et al. (1989).

**Origin, size, shape and Position of micronucleus.**

Micronuclei may arise from different types of chromosomal aberrations, and hence appear of different size & shape. Micronucleus is a small membrane bound chromatin mass, which may arise either from acentric or centric fragments of chromosome or chromatin materials due to choromosome breakage (structural chromosome damage) or whole chromosome leading to the production of numerical chromosome damage(Schmid, 1976). Usually one, very rarely two or many micronuceli per cell are observed attached to main nucleus or as a secondary nucleus in close viscinity to the main nucleus (Schmid, 1976). The size of micronuclues can distinguish the chromosome loss and chromosome breakage (Yamamoto and Kikuchi, 1980). Manna and Sadhukhan (1986) reported that the size of the

micronucleus appeared to be 20-25 times smaller than the main nucleus. Hose et al. (1987) noted that the usual shape of the micronucleus is round, almond or ovoid having a diameter of 1/20th to 1/10th of the erythrocyte nucleus, rarely approaching 1/3rd the size of the main nucleus in kelpbass. Carrasco et al. (1990) reviewed the different shapes of nuclear anomalies and micronuclei. The earlier hypothesis of different origin of micronucleus was confirmed by Gabriele et al. (1992) using CREST antibodies and fluorescent staining.

It is generally assumed that aneuploidogenic agents (eg. Colchicine) induce micronuclei containing whole choromosome which may be due to non-disjunction; while clastogenic agents(eg. Mitomycin-C) induce acentric micronuclei (Gabriele, et al., 1992).

By combined use of flow cytometry and antikinetochore antibody (CREST) staining, Nüsse et al. (1992 a) studied the DNA distribution and origin of micronucleus induced by chemicals. Jones and Parry (1992) and Miller and Nüsse(1993) explained the origin of micronucleus out of chromosomal damage during cell division by making use of fluorescence, in situ hybridization and antikinetochore antibody staining techniques.

## Erythrocytic Anomalies

Besides the micronucleated erythrocytes, enumeration of erythrocytic anomalies were also carried out by a few workers. Erythrocytic anomaly includes mainly changes in

nucleus structure termed as nuclear anomaly. These nuclear anomalies may arise out of cytotoxic effects of pollutants / chemicals (Katz, 1950; Hooftman and de Raat, 1982). Variation in blood cell constitution could be a useful indicator of pollution effect (Hunn and Greer, 1991). Carrasco et al. (1990) encountered different erythrocytic abnormalities in contaminated fish. However, it was less correlated to the body burden of suspected contaminants, leaving doubts regarding their origin. Cadmium exposure to Carassius auratus caused significant dose dependent impairment of erythropoietic capacity (Houston and Keen, 1984). Cadmium damages erythropoietic cells (Garofano and Hirshfield, 1983) and cause loss in integrity of cell membrane and membrane bound enzymes by enhancing lipid peroxidation (stacey and Kappus, 1982b; Younes and Siegers, 1984). Exposure of fish to heavy metals and other environmental pollutants cause different nuclear anomalies including micronucleus (Nikinmaa, 1990).

Erythrocyte counts vary according to age, sex and size in aquatic organisms (Siddiqui and Naseem, 1979). The normal blood cell structures itself may vary within and between species according to age, size environmental factors, etc. (Bhaumik et al., 1987; Tripathi, 1989; Folmar et al., 1992).

# MATERIAL AND METHODS

# MATERIAL AND METHODS

## Experimental animals

Endemic cichlid fish, Etroplus suratensis locally known as 'Karimeen' having the vernacular name as PearlSpot (or green chromide) is chosen as the test animal. It inhabits the brackishwaters and estuaries of India, Sri Lanka and Pakistan. In India, the major areas of distribution lie between Karnataka Coast on the west and up to Chilka lake, on the east. It feeds on detritus plankton, small aquatic insects, aquatic algae like Spirogyra and Oscillotoria. It is available throughout the year, but the peak season is January to April and September to November. They contribute a significant percentage of the landings in Chilka lake, Pulicat lake and Kerala backwaters (Raju et al., 1987).

Etroplus suratensis matures when attains a size of 10-12 cm. and grows to a maximum length of 25 cm. It responds positively to induced breeding and artificial feeding and hence it has a good potential for culture. It is euryhaline in its habitat and can be reared in the laboratory in static water with proper management.

Owing to these qualities and local abundance, it is selected as a test animal for the present studies.

## COLLECTION AND MAINTENANCE OF ANIMALS

Specimens of size ranging 70-90gm. with a mean weight 83.4 (S.D=7.1) were collected by cast net from fish ponds of Narakkal research centre of Central Institute of Brackishwater Aquaculture (CIBA) and Matsyafed fish farm, Narakkal. They were then acclimatized in a large tank or in a happa set in the natural water for about 3-4 days at the collection centre itself. Finally they were transported alive to the laboratory using 100 litre bins with least transporting stress.

In the laboratory the animals were kept in the same transported water for about 6 hours with sufficient aeration and then transferred carefully to the tank containing water of 4.0-5.0 ppt salinity. Animals were allowed to live under these conditions for about 20-25 days. Filtered tap water was used for dilution purposes. Temperature of water was 27.5 $\pm$ 1.5°C and pH was 7.0-8.0. Water was exchanged twice a day to avoid stress and to maintain cleanliness of the tank. Animals were fed regularly twice a day ad libitum with artificial pellet feed, prepared from fish and prawn meal and other conventional ingredients and with small prawns and clams. Sufficient care was taken to siphon off the excess food and waste materials to maintain hygienic conditions and thereby avoiding mechanical irritation, clogging of gills, turbidity and toxicity.

These maintenance procedures were strictly followed during both acclimation and experimental phases. No disinfectant was used to avoid fungal and microbial attack keeping in mind that they may also contribute to the cytotoxic effects in test animals.

TEST CHEMICALS

1. Colchicine: (Source : S.D. fine chem. Ltd., Boisar) Colchicine is a clastogenic, aneuploidogenic spindle poisoning alkaloid derived from the corm and seeds of the meadow saffron, Colchicum autumnale Linne. (Groliman, 1960). It is soluble in water.

2. Cyclophosphamide-Endoxan Asta : (Source: M.S. Khandelwal, Bombay)
Cyclophosphamide (Cp) is a water soluble compound and is cytotoxic, mutagenic, immunosuppressive, alkylating, nephrotoxic, anti-tumor agent (Rang and Dale, 1987).

3. Mitomycin-C (Source: Biochem Pharma, Ind., Bombay)
Mitomycin-C (MMC) is a aneuploidogenic, mutagenic anticarcinogenic antibiotic obtained from the culture broth of Streptomyces caespitosus (Rang and Dale, 1987) It is a water soluble direct mutagen.

4. Cadmium: (Source: Sarabhai M.Chem.,Baroda)
Cadmium is a non-essential cytotoxic element both in higher vertebrates and in fish (Gill and Pant 1985). It

is soluble in water. For the purity of the compound, $CdCl_3$ was calibrated to cadmium reducing the molecular weight of chloride.

## EXPERIMENTAL SET UP

### Base-line study

From Narakkal Matsyafed and CIBA fish ponds samples were collected and analysed for background micronucleus(MN) and nuclear anomaly Counts (NA). Three samplings were carried out from each station. Ten thousand cells per animal and six animals per site at a sampling time were analysed.

### Treatment

Three sublethal doses of each of aforesaid chemicals were given to the test animals whereas control animals were exposed to normal saline. The doses were determined on the basis of tolerance limits of each chemical used.

### Dose determination

Ninety six hour maximum tolerance dose for cyclophosphamide (200 ppm), Mitomycin-C (2 ppm), Colchicine (1 ppm), and Cadmium (2 ppm) was considered as the maximum dose in each case. Subsequent doses were fractions of the maximum dose. For each dose group twelve fish were randomly selected. Three animals were bled each time after 24 hrs, 48 hrs, 72 hrs,

and 96 hrs and a minimum of 2000 cells/animal were counted for micronucleus incidence. A summary of the experimental set up for treated animals is given in table 2.

Exposure to Genotoxicants

All the test solutions were prepared in Physiological normal saline (0.9% Nacl). A dose of 0.5-1.0 ml. of mutagenic solution was injected intraperitoneally using 1 ml. tuberculin syringe with No.1 needle. The uniformity of the mode of injection was maintained for all the mutagens. Anaesthesia was not used to narcotize the fish in order to avoid the influence of extraneous chemicals.

The fish were netted with least stress and swabbed carefully with 70% alcohol. Injection was given on the lateral region of belly inserting the needle obliquely in such a way that no organs were damaged. After injection the site was again swabbed with 70% alcohol and animals were replaced in the water tank.

Field study

Live specimens of E.suratensis were collected from the Narakkal Matsyafed fish pond (Control station), Narakkal waste water canal and Eloor industrial area (of Periyar river), Kochi and were analysed for genotoxicity.. Ten thousand cells per animal and six animals per site at a sampling time were analysed.

Table-2 Experimental set for animals treated with mutagens

| Mutagen | CP | | | Cont | Cadmium | | | Cont | MMC | | | Cont | Colchi-cine | | | Cont |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose (ppm) | 100 | 150 | 200 | - | 0.5 | 1.0 | 2.0 | - | 0.5 | 1.0 | 2.0 | - | 0.1 | 0.5 | 1.0 | - |
| No.of animals | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| No.of Animals bled after 24hrs | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 48hrs | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 72hrs | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 96hrs | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| No.of cells counted per animal | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 | 2000 |

CP - Cyclophosphamide, Cont-Control, MMC-Mitomycin-C.

Three samplings at an interval of fifteen days were carried out from each station and their mean value was taken for final comparison study.

**Blood collection**

Following the method of Hose et al. (1989), 0.06-0.125 ml of blood was collected from each individual by Cardiac puncture using a heparinized (4 mg/ml) 1 ml. syringe. The blood collection was also tried with sodium citrate (2%) solution as an anticoagulant. The site of puncture was cleared with 70% alcohol before and after the collection.

Slide preparation, processing and observation

Following methods were tried for slide preparation and staining before adopting the final one used for routine screening.

1. Schmid (1976)

Blood smears were air dried and placed in a clean dark slide box for a day for maturation and then stained with undiluted May-Gruenwald (S.d.fine) solution for 3 min and May-gruenwald diluted (1:1) with phosphate buffer (pH 6.8) for 2 min. Slides were washed in distilled water. Then they were counter stained in diluted Giemsa (Merck) with sorenson's phosphate buffer (1:4, pH 6.8) for 10 min. Rinsed in tap water and then in distilled water and finally were screened for micronuclei and nuclear anomalies.

2. Salamone et al. (1980)

Blood smears were air-dried, fixed in absolute methanol for 5 min, stained for 20 min in 5% solution of Giemsa in 0.01 M phosphate buffer (0.71 gm. $Na_2 HPO_4$ , 0.68 gm. $KH_2 PO_4$ 1 lit. distilled water, pH 6.8) and Screened for micronuclei and nuclear anomalies.

3. Hooftman and de Raat (1982)

Blood smears were prepared from peripheral blood and were fixed in methanol and stained in Feulgen (Merck) as follows. Hydrolyse in 1 N Hcl for 15 min at 60°C. Rinse with cold 1N Hcl and then with distilled water. Stain for 1 hr at room temperature in schiff's solution. Rinsed 3 times with freshly prepared bisulphite solution (10% $K_2 S_2 O_5$ , 5 ml nHcl to 100 ml dist. water). Counter stained for 1 min in a 1% aqueous light green (Himedia) solution. Rinsed with distilled water, dried and screened under oil immersion objective for nuclear lesions.

4. Hayashi et al. (1984)

Blood smear was dried over night, fixed in methanol for 5 min and stained with 3% Giemsa in sorenson's phosphate buffer (pH 6.8) for 25 min. Then the slides were screened for micronucleus and nuclear anomalies.

5. Walton et al. (1984)

Blood smears were fixed immediately in ethanol acetic acid (3:1) for 10 min., air dried, stained with 2% aceto-orcein (2 gm orcein, 45 ml glacial acetic acid to 55 ml sorenson's buffer, pH 6.8) for 3 min, washed with

distilled water and screened for micronucleus and nuclear anomalies.

6. Manna and Sadhukhan (1986)

Blood smears were stained with 5 drops of Wright stain (Qualigens fine chemicals) for 5 min after overnight maturation time. Stained slides were washed in distilled water, dried and screened for micronucleus and nuclear anomaly.

7. Hose et al. (1987)

Blood smears were fixed in absolute methanol for 15 min and were stained with May-Gruenwald-Giemsa (stock May-Gruenwald and 3% Giemsa) Slides were screened for micronucleus and nuclear anomaly.

8. Carrasco et al. (1990).

Blood smears were air-dried, fixed in absolute methanol for 25 min and stored in dark containers. Stained with stock Giemsa solution for 45 min and observed for micronucleus and nuclear anomaly.

9. Besides the aforesaid stains, 1% Toluidine blue ( Loba chemie ) and different gradation of Giemsa solution (3%, 5%, 10% , 20% and 40%) prepared in sorenson's buffer (pH 6.8) were also tried.

**Method developed for screening of micronucleus and nuclear anomaly.**

The method of Hayashi *et al*. (1984) was found to be good and followed in the experiment with little modifications. Briefly, blood smear was made on a acid washed clean slide by the standard smearing method (Lucky, 1977). The slides were then kept in a dark place to avoid light reactions. After air drying period the slides were kept for overnight maturation. These slides were fixed in absolute methanol for 10-15 min stained with freshly prepared and cloth filtered 20% Giemsa in Sorenson's buffer (pH 6.8) for 15-20 min in a coplin jar. After staining, the slides were washed in mild running tap water and then air dried after distilled water wash. Two slides per individual were prepared. Unmounted slides were screened for estimating the rate of micronucleus and nuclear anomaly incidences.

The dry stained slides were observed and screened under 1000 x magnification with oil immersion objective, through NIKON OPTIPHOT, No.1 (Japan), microscope equipped with a built-in automatic camera for microphotography. Six thousand erythrocytes per three individuals were observed and the micronuclei and nuclear anomalies finalised on pooling the data in a group (Metcalfe, 1988). The rate of micronucleus and nuclear anomaly formation was expressed as micronuclei and nuclear anomalies per 1000 cells. The total count of micronucleus and nuclear anomaly was denoted as

nuclear lesion count. Artifacts were excluded on the basis of base-line studies. Only non-refractile particles and intact cells were counted as abnormalities.

The size, shape, position and diameter of micronuclei and erythrocytes were analysed using stage and occular micrometer.

Statistical Analysis.

Two-Way analysis of variance was applied to test the significance of variation between doses and time in micronucleus and nuclear anomaly production for the test chemicals. Critical difference (C D ) analysis was carried out following two-way Anova to test the significance of variation between the pairs of doses and time.

# RESULTS AND DISCUSSION

## RESULTS & DISCUSSION

### Slide preparation and Micronucleus (MN) Count.

Various methods suggested by different workers (Schmid, 1976; Salamone et al., 1980; Hooftman and de Raat, 1982; Hayashi et al., 1984; Walton et al., 1984; Hose et al., 1987; Carrasco et al., 1990) were tried to prepare the smear for the erythrocytic micronucleus count and an optimum method was developed to get a good spread of cells and a clear contrast between the cytoplasm and nucleus.

Although both sodium citrate (2%) and heparin (4 mg/ml) prevented clotting effectively, heparin was found to be a better anticoagulant for the erythrocytic micronucleus test. Sodium citrate badly damaged the cells through hypotonic effect and led to more artifacts. Similar observation was made by Smit and Hattingh (1980) about the use of sodium citrate solution as an anticoagulant.

Giemsa staining for 15 minutes with a 20% working solution yielded good contrast between cytoplasm and nucleus of erythrocytes. Increased fixation and staining time and stain concentration did not have any effect.

Feulgen's method (Hooftman and de Raat, 1982) appeared to be cumbersome and gave no micronucleus in erythrocytes but at the same time micronuclei were observed using Giemsa Stain during the base-line studies. Similar observation was made by Hooftman and de Raat (1982), Das and Nanda (1986) and Majone et al. (1988) who reported lower micronucleus frequencies with Feulgen reagents than with Giemsa method. The reason being that the Feulgen stain causes drastic hydrolysis of material which yield free aldehyde group at nuclear DNA level and also lead to RNA removal (Overend and Stacey, 1949). Thus such a staining technique is specific for nuclear DNA. However, it is possible that the hydrolysis might destroy small DNA fragments like those present in micronucleus. Such staining techniques, causing drastic hydrolysis, of the material, are not suitable for micronucleus test. Toluidine blue (1%) stained the cells bright red and presented many stain particles and hence led to more artifacts. By Toluidine blue and Aceto-orcein good differential staining was not obtained.

May-Gruenwald (Stock) in combination with 20% Giemsa (Schmid, 1976) gave equally good results as with Giemsa alone. However, the aforesaid procedure was found to be time consuming. The staining procedure using Wright stain (Manna and Sadhukhan, 1986) did not give satisfactory results. The use of 15 days old stain, suggested by manufacturer (Qualigens fine chemicals, Bombay) also did not

improve the contrast between nucleus and cytoplasm of the cells.

The stained slides were generally observed without mounting since clearing of slides with xylene and mounting with DPX led to the gradual fading of stain. This was also observed by Dass (1990).

Base-line Study:

Erythrocyte Morphology:

The peripheral erythrocytes of E. suratensis were of the length ranging from 9.96 µm to 13.28 µm with mean 11.62 ± 1.16 µm). The cytoplasm appeared orange coloured whereas nucleus stained pink (Plate 1-9) with 20% Giemsa stain. The nucleus is clearly structured with a mean length of 3.4 ± 0.49 µm and has a well defined boundary (in contrast with ripe mammalian erythrocytes which have no nucleus). The cells were elliptical, oval or round in shape.

Nuclear lesions (Micronuclei + Nuclear abnormalities)

Consistent variations in the shape (smooth, elliptical) of the mature erythrocyte nucleus were noted in the pearlspot. These variations constituted the lesions of the nucleus in the form of micronucleus and nuclear anomaly.

The micronucleus consideration was given to the cells with clear and membrane bound nuclear mass, smaller than

the main nucleus (1/10th to 1/2.75 of main nucleus) but with similar colour intensity. However, most of the micronuclei ranged between ¼th to 1/3rd of main nucleus. Not more than one micronucleus per cell was noted (Plate.1). Mostly micronucleus was attached with main nucleus, and rarely separated micronuclei were noted. This is not in agreement with the observation made by Manna and Sadhukhan (1986), who reported that the micronucleus is 1/20 to 1/10th of main nucleus in size and occured one to eight per branchial (gill) cell of tilapia. Similarly Hose et al. (1987) reported that the micronucleus diameter size ranged from 1/20th to 1/10th of the main nucleus of erythrocyte, rarely approaching 1/3rd of the parent nucleus in white croaker and kelp bass. The difference in size of the micronucleus noted in this study might be due to the difference in tissue type and species being tested. Bhaumik et al. (1987) and Tripathi (1989) have also opined that different species and tissues may have different nuclear content and thus different sized micronucleus in erythrocytes.

Different types of nuclear anomalies noted in erythrocytes are shown in plates 3 to 5. The nuclear anomalies constituted blebbed nucleus, lobed nucleus and bilobed nucleus.

The 'blebbed nucleus' was a relatively small evagination of the nuclear envelope which ranged in shape from a slight protrusion to a stalked structure (Plate.4)

The 'lobed nucleus' was considered on the basis of the nuclear evagination, longer than the blebbed ranging from simple swellings of the nuclear surface to the presence of multiple lobes etc. (Plate 3).

The 'bilobed nucleus' as shown in plates 4 and 5, is nuclear strcutre constricted at centre giving the nucleus a dumbbell shape. It was differentiated from micronucleus by the depth of the invagination at centre. One half invagination of the nucleus represented micronucleus whereas lesser than half represented bilobed nucleus. The nuclear anomalies observed in this study were similar to those observed by Carrasco et al. (1990) in White croaker (Genyonemus lineatus) erythrocytes.

**Base-line Micronucleus+Nuclear Anomaly incidences:**

The micronucleus and nuclear anomaly frequencies observed during the base line studies, are given in Table 6. The base-line data or background reading for micronuclei and nuclear anomalies were found to be low and vary within a range of 0.0 - 0.3 and 0.2 - 0.6 per thousand cells respectively (Table-3).

Table 3: Base-line micronucleus and nuclear anomaly incidences in control stations

| Area | Sampling Period | Frequency MN (o/oo) | NA (o/oo) | Total (o/oo) |
|---|---|---|---|---|
| Matsyafed fish pond, Narakkal. | March (III week) | 0.2 | 0.6 | 0.8 |
| | May (I week) | 0.1 | 0.5 | 0.6 |
| | May (II week) | 0.3 | 0.6 | 0.9 |
| CIBA Fish Pond, Narakkal | May (I week) | 0.2 | 0.2 | 0.4 |
| | May (II week) | 0.0 | 0.4 | 0.4 |
| | June (I week) | 0.2 | 0.5 | 0.7 |

MN - Micronucleus

NA - Nuclear Anomaly

This low range of base line nuclear lesions were also observed by Hose et al. (1987) and Dass (1990) in erythrocytes of white croaker, kelp bass and in Pearlspot respectively.

These so-called 'Natural' or background incidence may interfere with the detection of pollutant mediated DNA damage. It is always desirable to have the test animal with low background nuclear lesions for micronucleus test.

Artifacts

The direct comparison of micronucleus with main nucleus helped in identifying the different artifacts. In the present study the artifacts were observed in the form of overlapping cells, ruptured cells due to hypotonic effects and stain particles as shown in plates 5 to 7. Stain particles and acidic materials other than chromosome fragments were main intervening artifacts which were excluded on focussing due to their refractile nature while comparing with main nucleus.

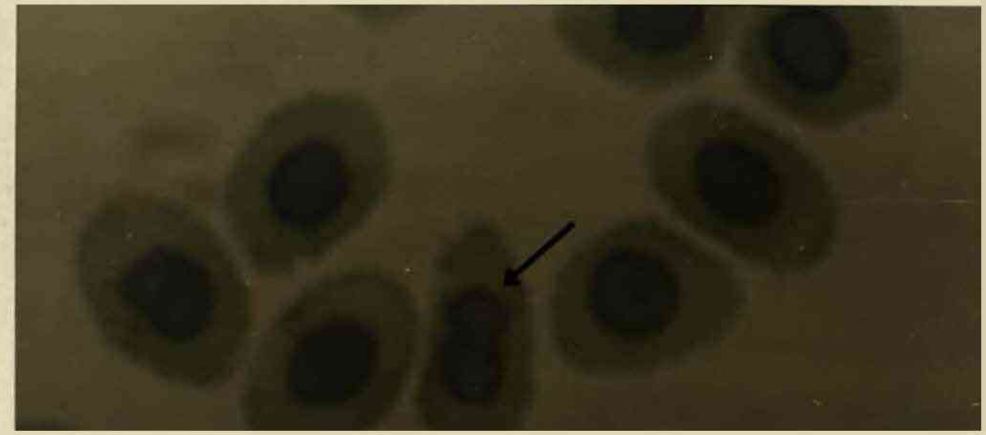
Genotoxic effects of chemicals.

The genotoxic effects of selected chemicals, viz. Colchicine, Cyclophosphamide, Mitomycin-C and Cadmium were studied in E. suratensis in the form of nuclear lesions comprising micronucleus & nuclear anomaly. The effect of dose & time of exposure on micronucleus and nuclear anomaly incidences were analysed by two-way Anova and is shown in Table-5,7,9,11 and in Fig. 6-9.

## *PLATE - 1*

*Upper - Arrow indicates erythrocyte with Micronucleus.*

*Lower - Arrow indicates erythrocyte with Micronucleus.*



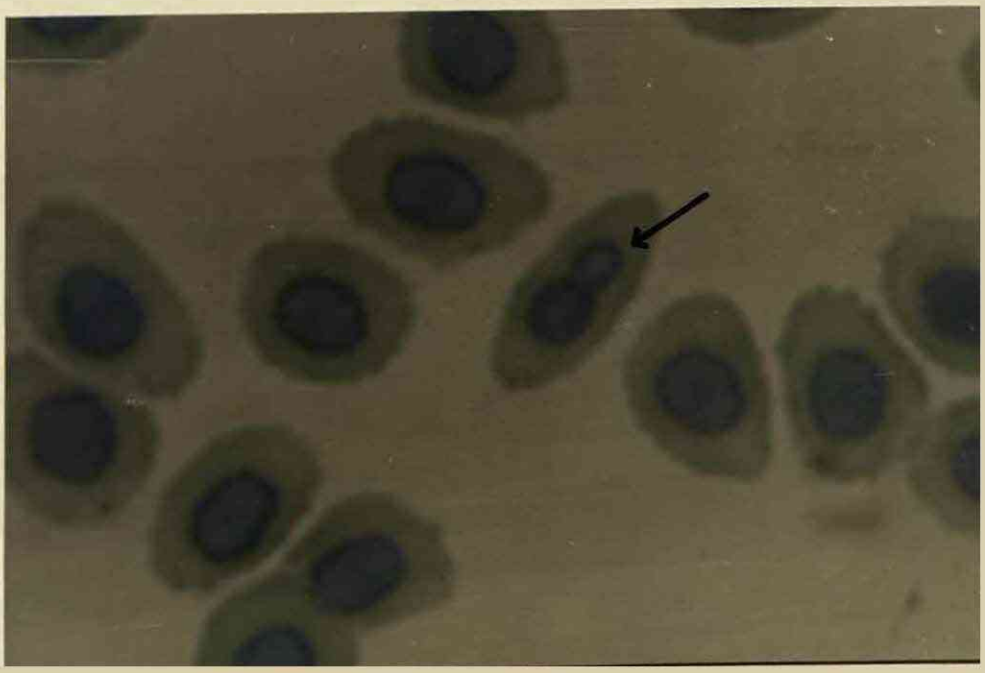
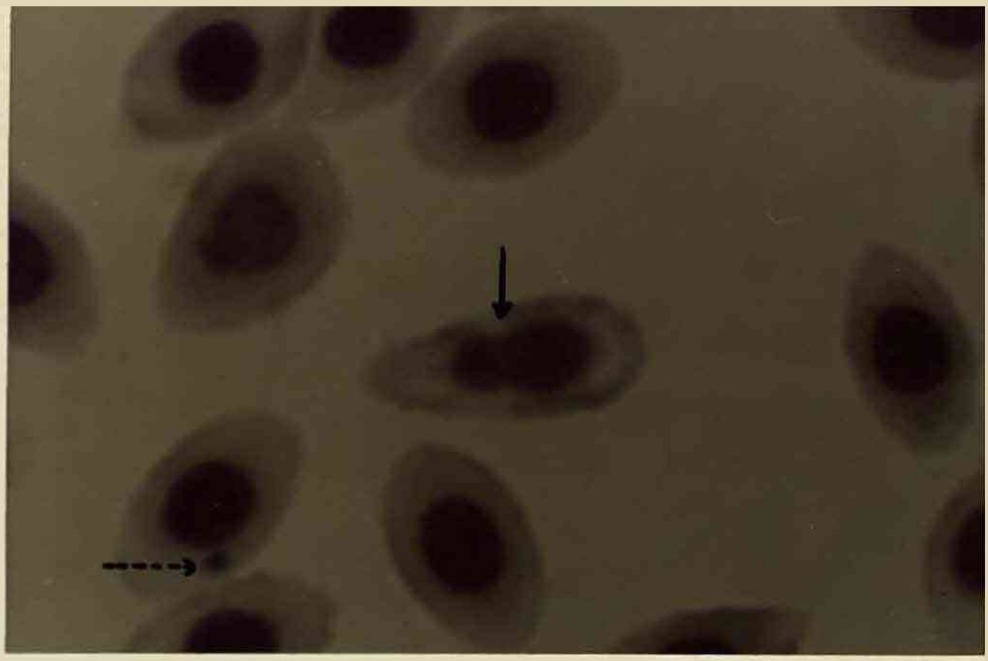
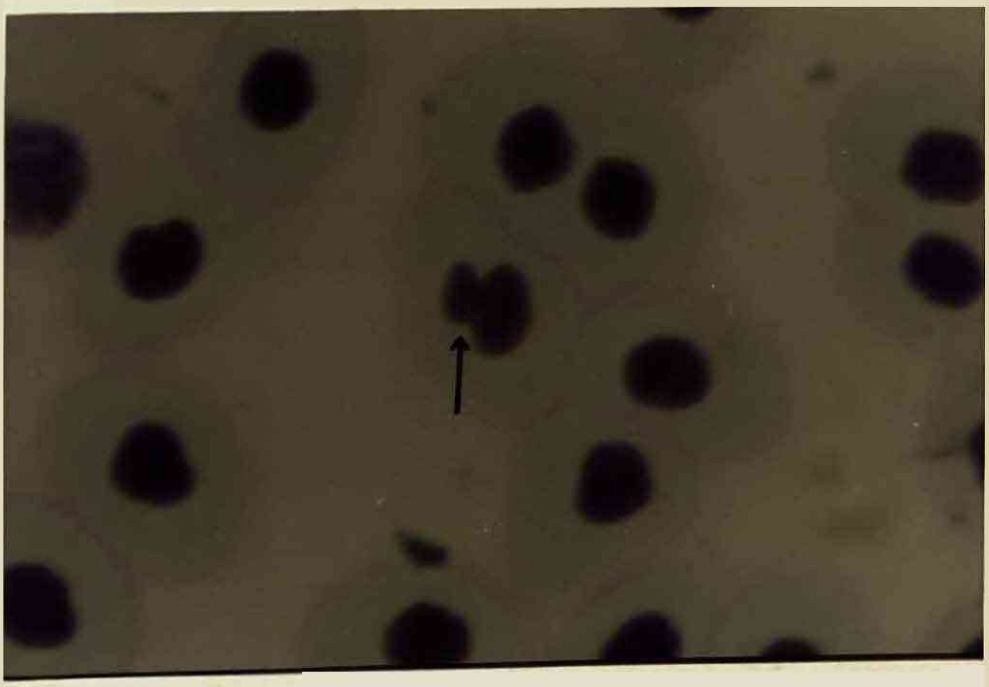


PLATE - 2

Upper - Arrow indicates erythrocyte with Micronucleus.

Lower - Arrow indicates erythrocyte with Micronucleus.

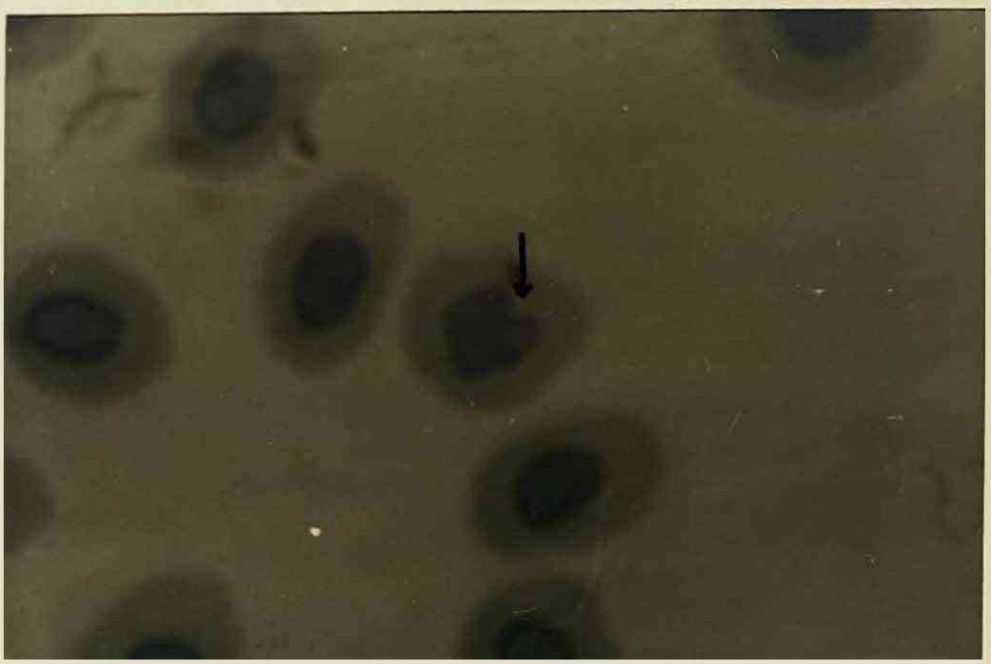




## *PLATE - 3*

*Upper - Arrow indicates erythrocyte with lobed Nucleus.*

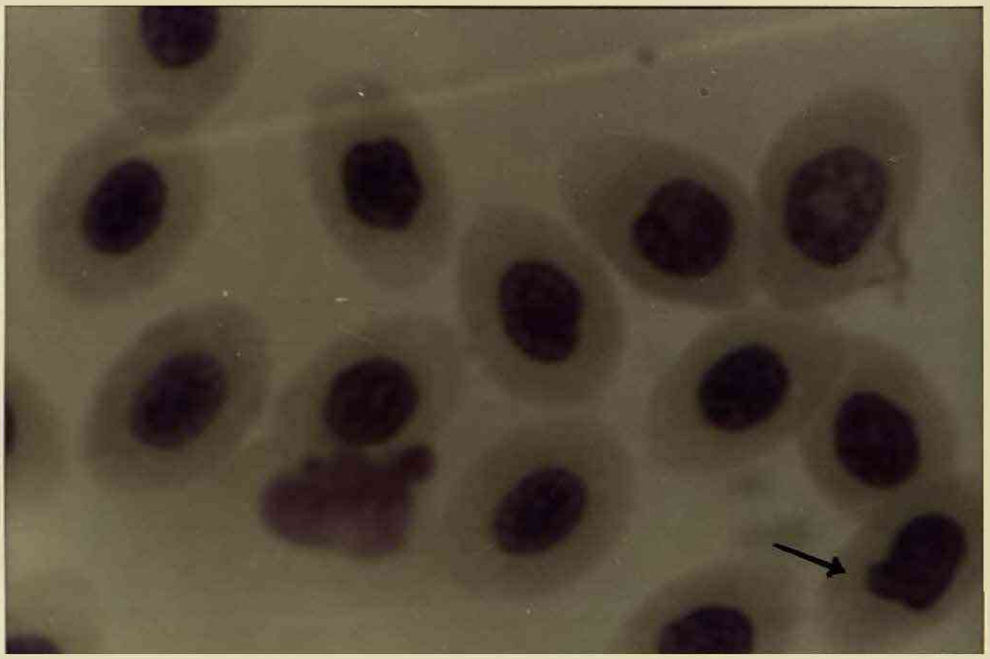
*Lower - Arrow indicates erythrocyte with lobed Nucleus.*

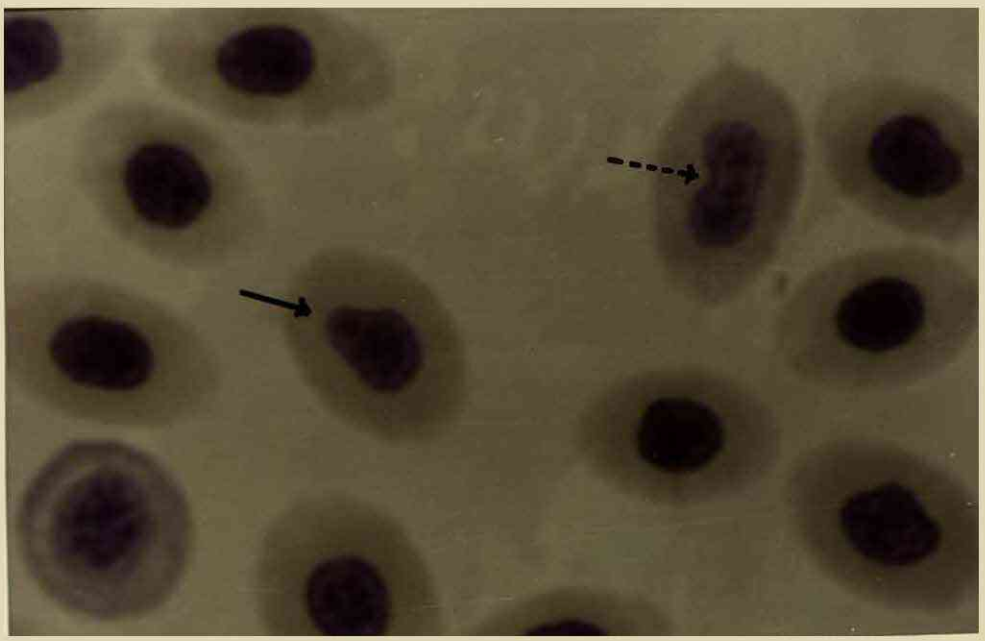


## PLATE - 4

Upper - Arrow indicates erythrocyte with blebbed Nucleus.

Lower - Solid arrow indicates erythrocyte with blebbed Nucleus.
- Brocken arrow indicates erythrocyte with bilobed Nucleus.

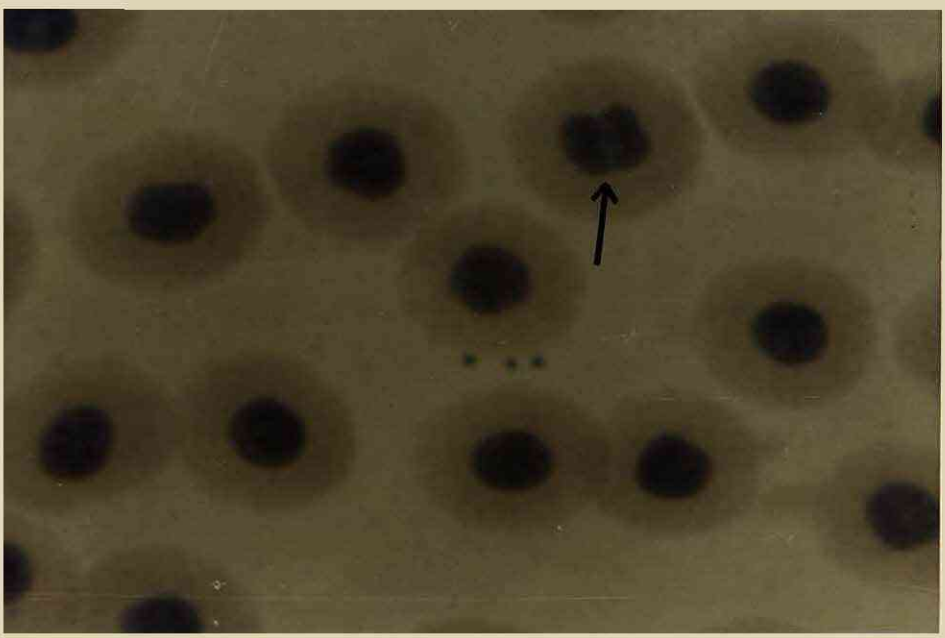




## *PLATE - 5*

*Upper - Arrow indicates erythrocyte with bilobed Nucleus.*

*Lower - Arrow indicates erythrocyte with bilobed Nucleus.*



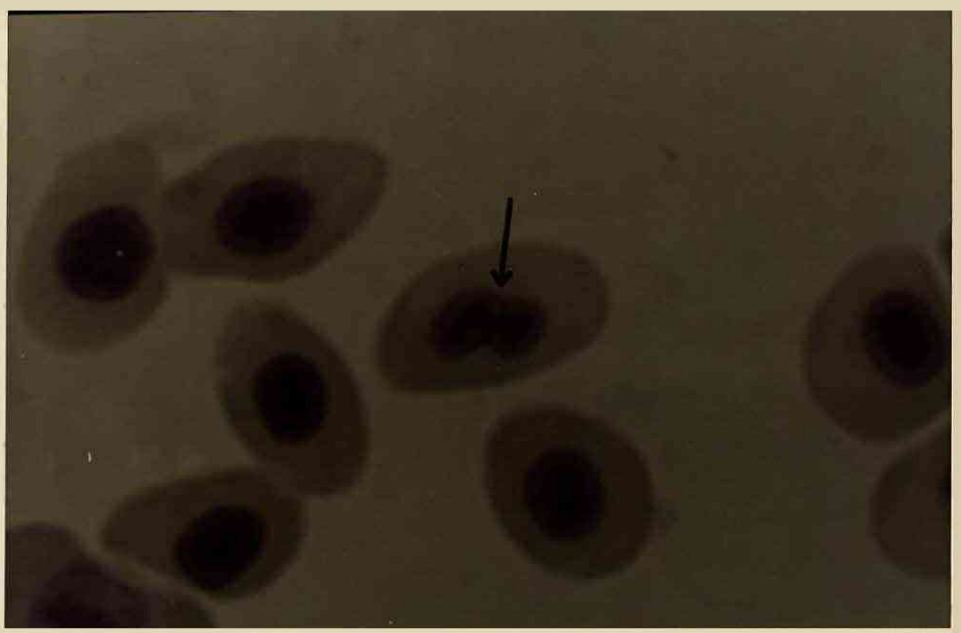
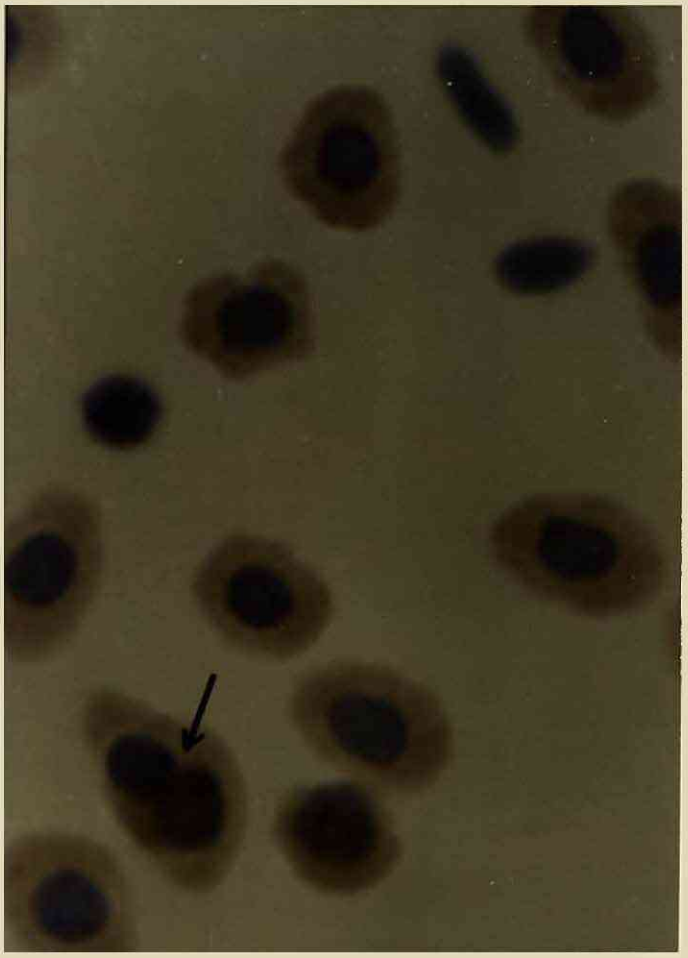


***PLATE - 6***

***Arrow indicates erythrocytes overlapping resembling Bilobed Nucleus.***



## *PLATE - 7*

*Upper - Arrows indicate deshaped erythrocytes showing Hypotonic treatment*

*Lower - Arrows indicate ruptured erythrocytes showing Hypotonic treatment.*

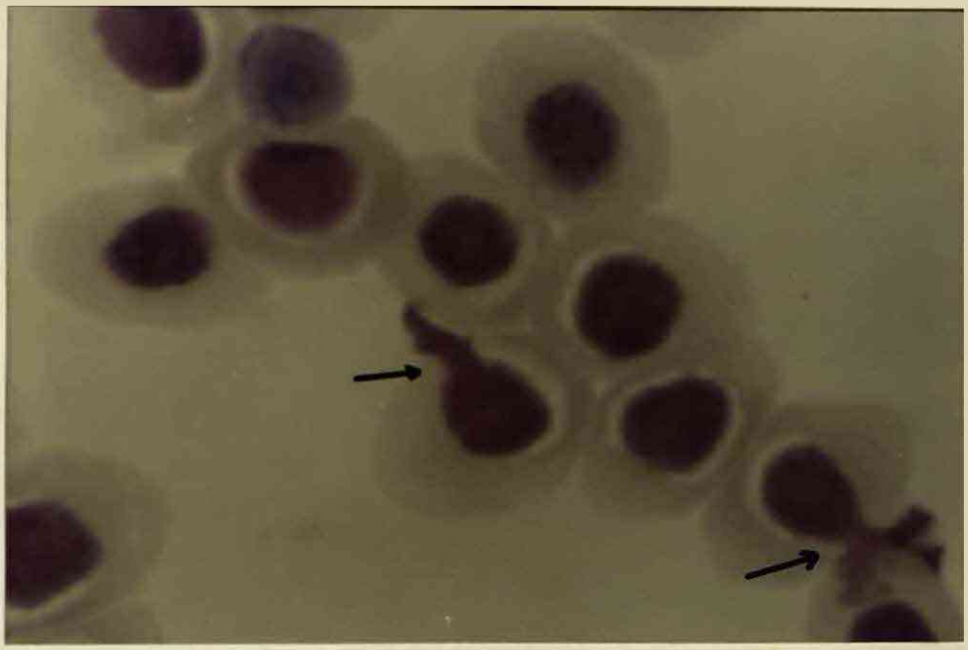
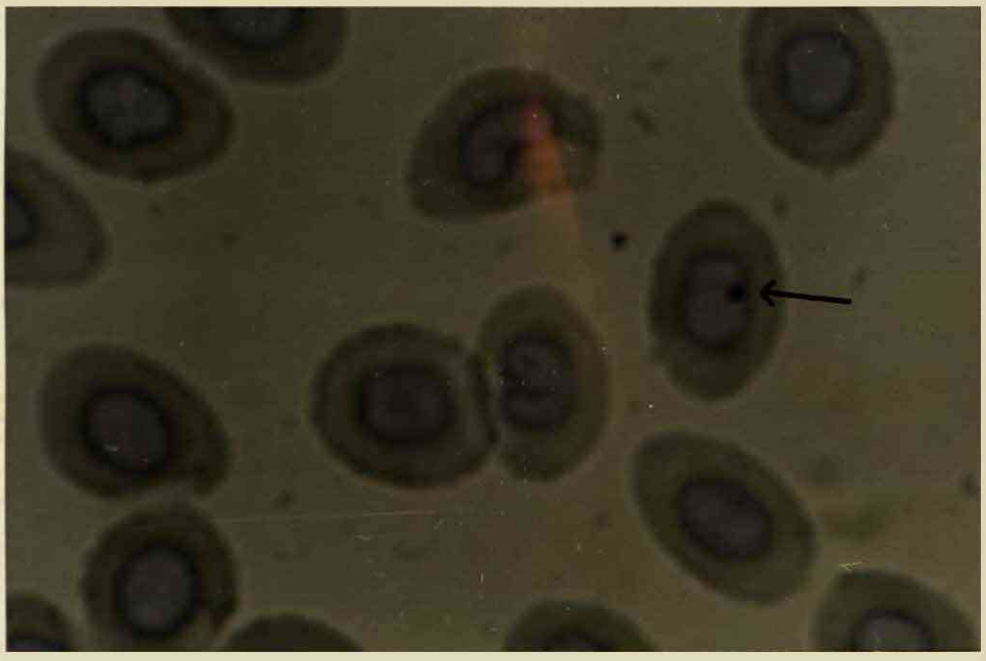
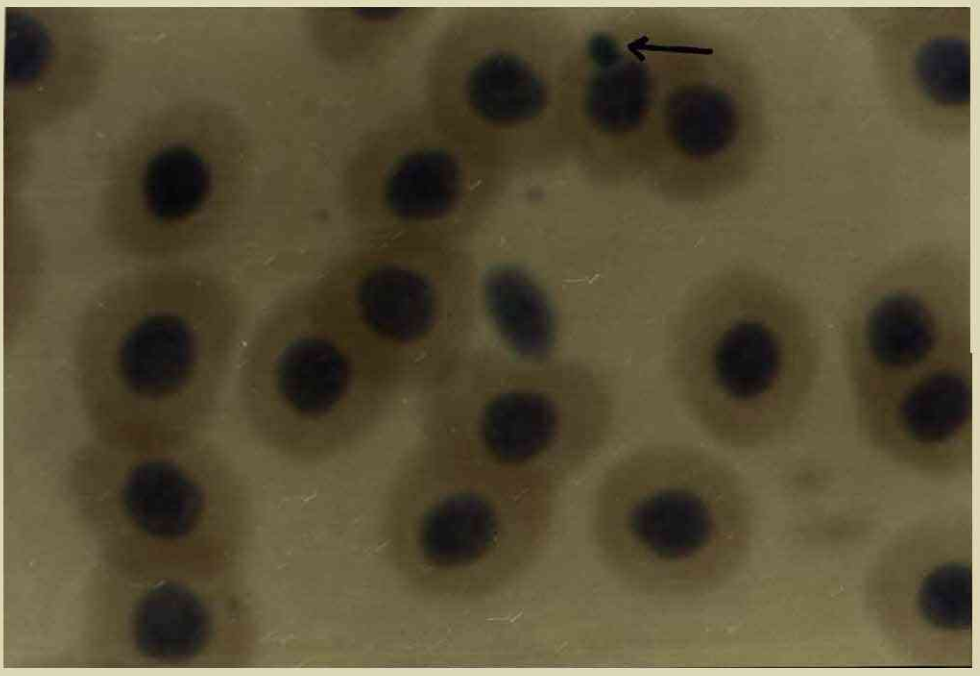


*PLATE - 8*

*Upper - Arrow indicates erythrocyte with stain particle resembling Micronucleus.*

*Lower - Arrow indicates erythrocyte with stain particle. resembling Micronucleus.*





Colchicine

Incidences of micronucleus, nuclear anomaly and nuclear lesion observed, in the animals exposed to different dose-levels of Colchicine for different periods, has been given in Table 4. At each level of dose, the frequency (0/00) of micronuclei, nuclear anomalies in treated animals were found higher than that of control animals. The frequency of nuclear lesions (MN + NA) increased with the increase of dose level and time of exposure (Table-4). The increase in micronucleus and nuclear anomaly frequencies with the increase in dose and time of exposure is shown in Fig. 6.

Two-way analysis of variance showed that the level of doses (0 , 0.1, 0.5 and 1.0 ppm) had significant effect ($P<0.01$) on micronucleus, nuclear anomaly and nuclear lesion incidences. Whereas time (24, 48, 72 and 96 hrs) had significant effect ($P<0.05$) on nuclear anomaly and nuclear lesion incidences but not on micronucleus (Table 5). Critical difference (C D) analysis showed that the variation between the doses 0 & 1.0 ppm was highly significant ($P<0.01$) both in micronucleus and nuclear anomaly production followed by 0.1 & 1.0 ppm in micronucleus production. In the remaining combination of doses and time variations observed were not significant. In total, the nuclear lesions, (MN + NA) production

Table 4: Frequency distribution (o/oo) of micronucleus, nuclear anomaly and nuclear in animals exposed to colchicine.

| Treatment | Dose (ppm) | Frequencies | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hr | | | 48 hr | | | 72 hr | | | 96 hr | | |
| | | MN | NA | NL | MN | NA | NL | MN | NA | NL | MN | NA | NL |
| Control | - | 0.2 | 0.4 | 0.6 | 0.2 | 0.6 | 0.8 | 0.2 | 0.6 | 0.8 | 0.2 | 0.6 | 0.8 |
| Colchicine | 0.1 | 0.4 | 0.6 | 1.0 | 0.4 | 1.4 | 1.8 | 0.6 | 1.4 | 2.0 | 0.6 | 1.4 | 2.0 |
| | 0.5 | 0.8 | 1.2 | 2.0 | 1.0 | 1.6 | 2.6 | 1.0 | 2.0 | 3.0 | 1.6 | 2.8 | 4.4 |
| | 1.0 | 0.8 | 1.6 | 2.2 | 1.4 | 1.8 | 3.2 | 2.0 | 2.8 | 4.8 | 3.2 | 3.6 | 6.8 |

MN-Micronucleus, NA-Nuclear Anomaly, NL-Nuclear lesion.

Table 5: Effect of dose and time of exposure to Colchicine on micronucleus, nuclear anomaly and nuclear lesion.

| Source | D.F. | F-Value | | |
|---|---|---|---|---|
| | | MN | NA | NL |
| Dose | 3 | 9.73** | 15.30** | 12.18** |
| Time | 3 | 2.43 | 5.39* | 3.94* |
| Error | 9 | | | |

** Significant at 1% level (P<0.01), * Significant at 5% level (P<0.05)

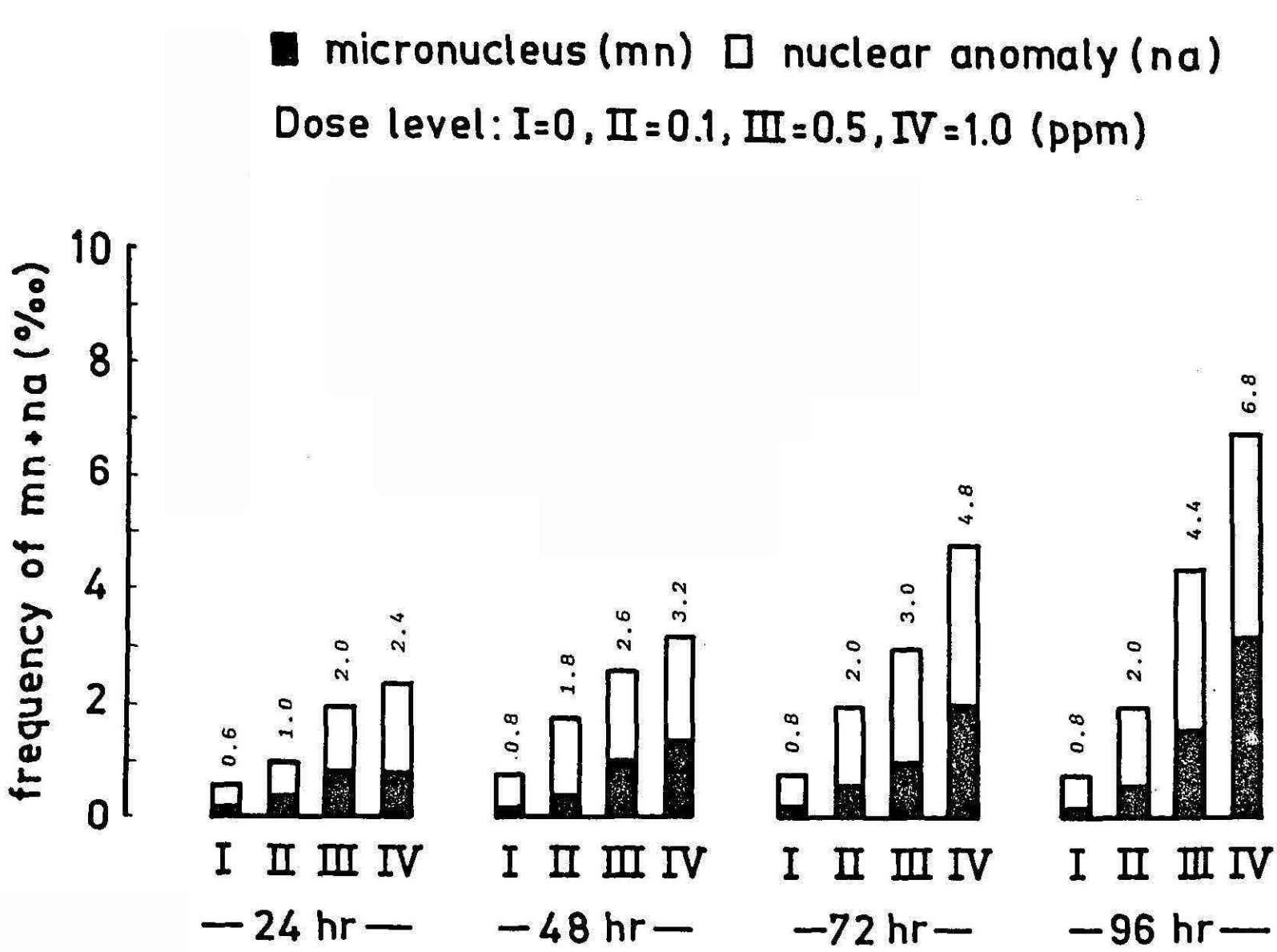



Fig. 6. TREATMENT WITH COLCHICINE

differed significantly between doses ($P<0.01$) and time ($P<0.05$). The C.D. analysis showed that the variation between the doses were in the order of 0 & 1.0, 0.1 and 1.0, and 0 and 0.5 ppm.

Dose dependent micronuclei and nuclear anomalies were produced by colchicine. However, a marginal increase noted between first sampling time (24 hrs.) and last sampling time (96 hrs.) for 0.1 and 0.5 ppm doses were not significant. This finding of no significant effect of time on micronucleus production may be due to the direct action of colchicine on microtubules causing metaphase arrest after administration. After exposure, most cells might have arrested at metaphase stage and only a few cells would have reached the end of mitosis in order to manifest micronucleus. After some time, the arrested cells might have resumed their mitotic activity and the frequency of micronuclei rose accordingly with marginal increase. Hence there might be a check in micronucleus induction and release over time as suggested by Majone et al. (1990). It is clear from the critical difference analysis that the differences between 24 hr and 48 hr, 24 hr and 72 hr and 24 hr and 96 hr was less, high and higher, though there was no significant variation between them. This may expalin the marginal increase of micronuclei over time.

From the result it is apparent that colchicine which is a representative of clastogenic agents, caused damage to the erythrocyte nucleus of E. suratensis giving rise to micronucleus and nuclear anomaly. This may be due to the spindle disturbing nature of colchicine and its effect on microtubules and thus resulting in failure of chromosome segregation.

The present observation is in agreement with Majone et al. (1990) and Gabriele et al. (1992), who have studied the effect of colchicine on micronucleus induction in mussel. They found significant increase in micronucleated cells up to 96 hr after the end of treatment with a hike in micronucleus incidence after 24 hr due to the release of metaphase arrested cells with fragments of chromatin materials resembling micronucleus.

Further, the increased rate of micronucleated erythrocytes in the present observation agreed with the findings of Flower et al. (1980) who reported that colchicine and its metabolites were found to be absorbed rapidly into the plasma and distributed in an apparent space wider than that of the body water and exert crucial effects on biological system by arresting the cell division. Similar positive genotoxic effect to colchicine was also observed in mammals (Groliman, 1960; Schmid, 1976;

Athwal and Sandhu, 1985; Sutou et al., 1986) as well as in aquatic organisms (Gabriele et al., 1992).

Cyclophosphamide (CP)

Frequency estimates (0/00) of micronucleus, nuclear anomaly in control animals and in the animals treated with 100, 150 and 200 ppm of cyclophosphamide, for different periods (24, 48, 72 and 96 hrs), are shown in table 6. It is obvious from this table that the treated animals had higher frequencies of nuclear lesions than that of control animals. Increase in nuclear lesions with increase in dose of cyclophosphamide and time of exposure, has been shown in Fig. 7. Cyclophosphamide induced both dose and time dependent micronucleus and nuclear anomaly production in pearlspot. Maximum frequency of micronucleus and nuclear anomaly was observed in the animals exposed to highest dose (200 ppm) for 96 hrs, (table 6). In other animals exposed to lower level of doses and for lesser time, the frequencies of micronucleus and nuclear anomaly production were higher than that of control animals.

Significant variation ($P<0.01$) between doses (0,100,150 and 200 ppm) and time of exposure (24, 48, 72 and 96 hrs) was observed in nuclear lesion (MN + NA) production. Both dose and time had significant effect ($P<0.01$ and $P<0.05$ respectively) on the micronucleus and nuclear anomaly production (table 7). Critical difference analysis showed a maximum significant variation in nuclear lesion incidences between 0 and 200, followed by 0 and 150,

Table 6: Frequency distribution (o/oo) of micronucleus, nuclear anomaly and nuclear lesion in animals exposed to cyclophosphamide.

| Treatment | Dose (ppm) | Frequency | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hr | | | 48 hr | | | 72 hr | | | 96 hr | | |
| | | MN | NA | NL | MN | NA | NL | MN | NA | NL | MN | NA | NL |
| Control | - | 0.2 | 0.4 | 0.6 | 0.2 | 0.4 | 0.6 | 0.4 | 0.4 | 0.8 | 0.4 | 0.4 | 0.8 |
| Cyclophosphamide | 100 | 0.8 | 1.2 | 2.0 | 1.8 | 0.8 | 2.6 | 2.4 | 3.0 | 5.4 | 2.4 | 3.0 | 5.4 |
| | 150 | 1.2 | 1.6 | 2.8 | 2.0 | 1.6 | 3.6 | 2.8 | 3.0 | 5.8 | 2.8 | 3.8 | 6.6 |
| | 200 | 2.0 | 1.8 | 3.8 | 2.2 | 3.2 | 5.4 | 3.0 | 4.2 | 7.2 | 3.8 | 4.8 | 8.6 |

MN - Micronucleus, NA - Nuclear anomaly, NL-Nuclear lesion.

Table 7: Effect of Dose and time of exposure to cyclophosphamide on micronucleus, nuclear anomaly and nuclear lesion.

| Source | D.F | F-Value | | |
|---|---|---|---|---|
| | | MN | NA | NL |
| Dose | 3 | 29.44** | 15.84** | 25.07** |
| Time | 3 | 9.31** | 5.53* | 9.47** |
| Error | 9 | | | |

** Significant at 1% level (P<0.01), * Significant at 5% level (P<0.05)

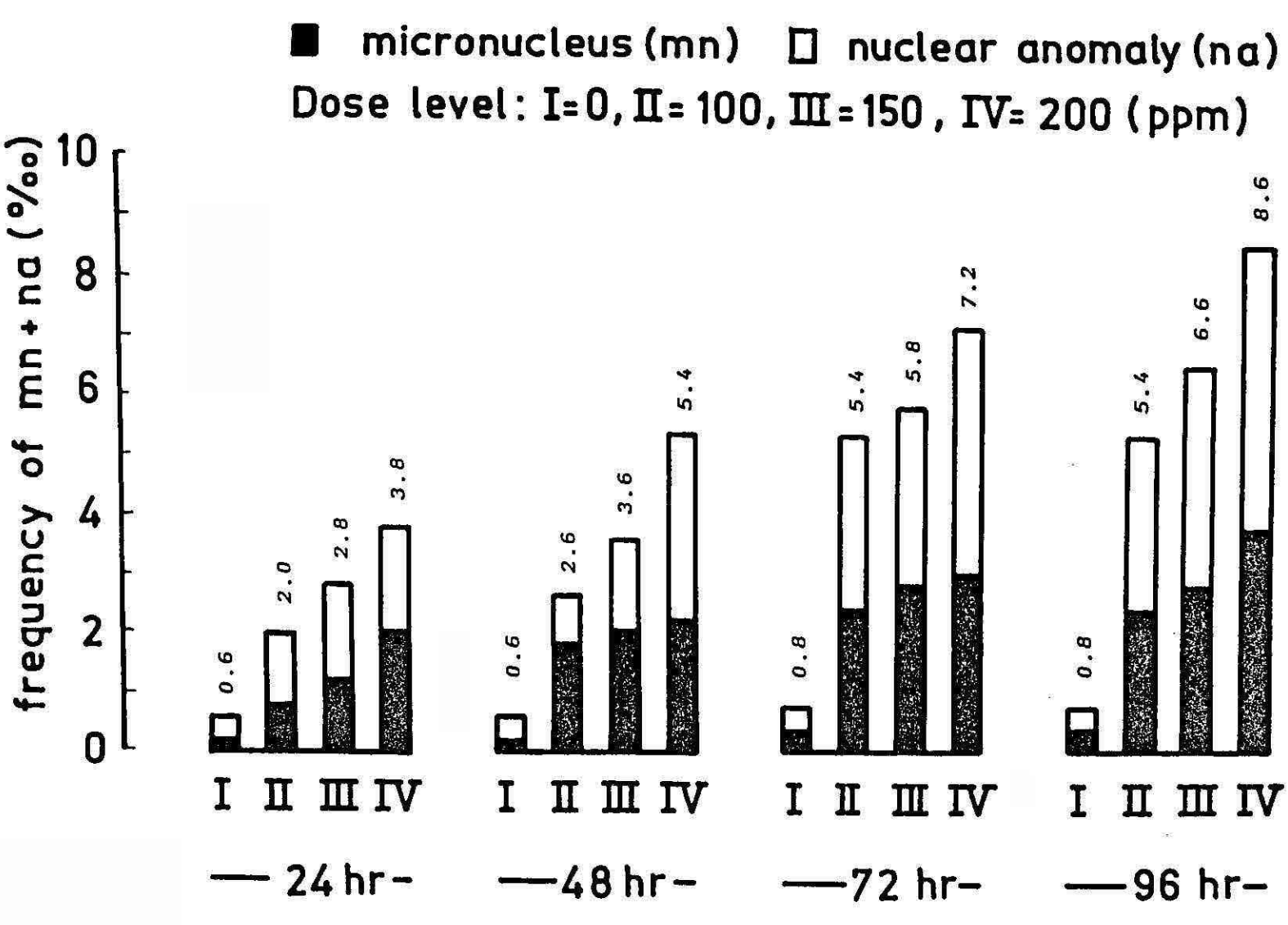



Fig. 7. TREATMENT WITH CYCLOPHOSPHAMIDE

0 and 100 and 100 and 200 ppm for dose and 24 and 96 hr followed by 24 and 72 hr and 48 and 96 hr for time. Similarly the C.D analysis for micronucleus and nuclear anomaly showed that the variation between doses were in the order of 0 and 200 ppm, 0 and 150ppm, 0 and 100 ppm and 100 and 200 ppm and the variation between time of exposure were in order of 24 and 96 hrs 24 and 72 hrs for micronucleus production. In rest of the dose and time combinations there were no significant variation in micronucleus production.

The effective response of cyclophosphamide noted in the present investigation might be due to the bio-activation of the promutagen (CP) by microsomal enzyme system as suggested by Kligerman (1979C) in Umbra limi and by Baksi and Means (1989) in Morone saxitilis larvae. The present observation was in agreement with the reports of Means et al. (1988), who studied the effect of cyclophosphamide on embryos of fish Morone saxitilis and Cyprindon varigatus and in the adults of Fundulus heteroclitus. Baksi and Means (1989) also observed the dose related genotoxic effects of cyclophosphamide on Morone saxitilis larvae. However, Dass (1990) reported incidence of micronucleus in E. suratensis with cyclophosphamide were not significant. This difference in observation may be due to the difference of age and size of the test animals. The animals selected for the present

study were adult specimens (mean 83.4 gm and S.D.=7.1), whereas animals selected by Dass (1990) were juveniles. Who observed that the 96 hr maximum tolerable dose of cyclophosphamide for E. suratensis was 100 ppm. Whereas in the present study the same was 200 ppm. Since the chromosomal damage or cellular death is the result of toxicity, it is to be expected that significant levels of micronuclei will only be found at toxic or subtoxic doses (Heddle et al., 1983). Hence, highest tolerable dose should be used for effective micronucleus induction. However between the present study and that of Dass (1990), the maximum tolerable doses are differing which may be due to age and size of animal. Similar differences in response, due to different size groups of a species to a mutagen agrees with the findings of Laprieno (1980) and Brunetti et al., (1992). This again, might be due to the difference of erythrocyte kinetics in the species at different age groups. The difference of micronucleus incidences in jeveniles and adults may explain that more number of erythrocytes are produced in adults than juveniles in E. suratensis. This is supported by the report of Siddiqui and Naseem (1979) who studied the erythrocyte production at different stages and in different species. They observed more number of erythrocytes in larger fish and at maturity stages in Labeo rohita and also in Tilapia zilli and Cyprinus carpio. Besides, the erythrocyte kinetics, the differential microsomal enzyme

production for cyclophosphamide metabolism is also attributed to the intraspecies variation in micronucleus production. In adult specimens, more production of Microsomal P-450 enzyme might have resulted in increased micronucleus production as suggested by Binder et al. (1984). it is further supported by the report of Bühler and Williams (1988) that the metabolism of any Xenobiotic may be influenced by sex, age, metabolic state, size and various environmental factors.

The positive genotoxic effects to cyclophosphamide (alkylating agent) observed in fishes including E. suratensis, were similar to the effects observed in mammals (Schmid, 1976; Evans, 1976; Mohn and Ellenberger, 1976; Salamone et al., 1980; Athwal and Sandhu, 1985; Gupta, 1989) and in aquatic organisms (Kligerman, 1979c; Kligerman et al., 1985. Hoeven et al., 1982).

Mitomycin-C (MMC)

Frequency distribution of micronucleus, nuclear anomaly and nuclear lesion, in animals exposed for 24, 48, 72 and 96 hrs to the different doses (0.5, 1.0 and 2.0 ppm) of mitomycin-C, has been given in table 8. Treated animals showed higher incidences of micronucleus, nuclear anomaly than the control animals. An increasing trend in nuclear lesions, with the increase in dose and time of exposure, is shown diagramatically in fig.8.

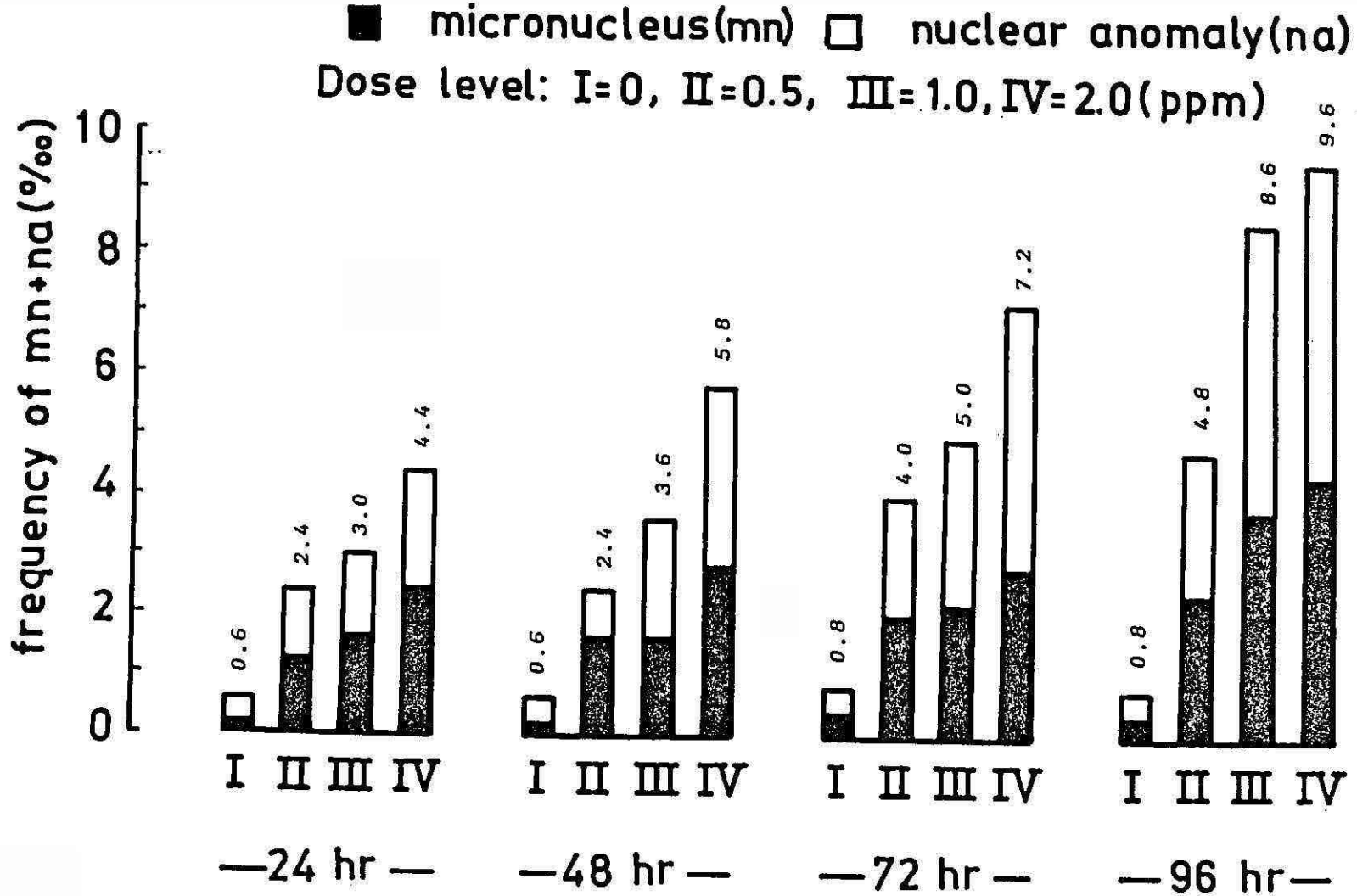



Fig. 8. TREATMENT WITH MITOMYCIN-C

Variation between doses (0, 0.5, 1.0 and 2.0 ppm) and time (24, 48, 72 and 96 hrs) was significant ($P<0.01$) in micronucleus production (Table 9). C D analysis showed that the variation between the doses were in the order of 0 and 2.0, 0 and 1.0, 0 and 0.5, 0.5 and 2.0 ppm and between the time 24 and 96 hr, 48 and 96 hr. In rest of the dose and time combinations there were no significant variation in micronucleus production.

Significant variation between dose ($P<0.01$)&time ($P<0.05$) was noted in nuclear anomaly production. According to C D analysis, maximum significant variation was observed between 0 and 2.0 ppm, followed by 0 and 1.0, 0.5 and 0.2, 0 and 0.5 and 0.5 and 1.0 ppm for dose and between 24 and 96 hr followed by 48 and 96 hr and 24 and 72 hr for time.

In total, the nuclear lesions production varied significantly between dose ($P<0.01$) and time ($P<0.05$). C D analysis showed that the variation between the doses were in the order of 0 and 2.0, 0 and 1.0, 0.5 and 2.0 and 0 and 0.5 for dose and between the time 24 and 96, 48 and 96 hr.

Intraperitoneal injection of mitomycin -C induced a dose and time dependant micronucleus and nuclear anomaly with linear increase in E. suratensis. Maximum nuclear lesions were obserbed with high dose and in last sampling time (fig.8).

Table 8: Frequency distribution (o/oo) of micronucleus, nuclear anomaly and nuclear lesion in animals exposed to mitomycin-C

| Treatment | Dose (ppm) | Frequency (o/oo) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hr | | | 48 hr | | | 72 hr | | | 96 hr | | |
| | | MN | NA | NL | MN | NA | NL | MN | NA | NL | MN | NA | NL |
| Control | - | 0.2 | 0.4 | 0.6 | 0.2 | 0.4 | 0.6 | 0.4 | 0.4 | 0.8 | 0.4 | 0.4 | 0.8 |
| Mito-mycin-C | 0.5 | 1.2 | 1.2 | 2.4 | 1.6 | 1.2 | 2.4 | 2.0 | 2.0 | 4.0 | 2.4 | 2.4 | 4.8 |
| | 1.0 | 1.6 | 1.4 | 3.0 | 1.6 | 2.0 | 3.6 | 2.2 | 2.8 | 5.0 | 3.8 | 4.8 | 8.6 |
| | 2.0 | 2.4 | 2.0 | 4.4 | 2.8 | 3.0 | 5.8 | 2.8 | 4.4 | 7.2 | 4.4 | 5.2 | 9.6 |

MN- Micronucleus, NA- Nuclear Anomaly, NL- Nuclear Lesion.

Table 9: Effect of dose and time of exposure to Mitomycin-C on micronucleus, nuclear anomaly and nuclearlesion.

| Source | D.F. | F-value | | |
|---|---|---|---|---|
| | | MN | NA | NL |
| Dose | 3 | 27.03** | 14.41** | 20.80** |
| Time | 3 | 7.44** | 5.45* | 6.93* |
| Error | 9 | | | |

** Significant at 1% level (P<0.01)

* Significant at 5% level (P<0.05)

In the present study, low but significant number of micronuclei and nuclear anomalies might be due to the cells that are gradually replaced the pre-existing ones. This low frequency might be due to the inhibition of mitotic activity in cells by mitomycin-C as stated by Das and Nanda (1986). However, a significant increase in micronucleus frequency with increase in exposure time might be due to high number of damaged cells undergoing mitosis enabling production of micronucleus and its accumulation over a period of time. This was also observed by Majone et al. (1988) in mussel.

The positive response of mitomycin-C observed in this study was in agreement with the reports of Gabriele et al. (1992) and Williams and Metcalfe (1992) who qualified mitomycin-C as an alkylating and aneuploidogenic genotoxic agent in aquatic organisms, through micronucleus test. Similar results, with other end points such as sister chromatid exchange and chromosome aberration are reported by Krishnaja and Rege (1982) and Kocan et al. (1982).

Increased micronucleus incidences might be due the action of free radicals generated from mitomycin-C, either through cross linking of DNA, prevention of DNA synthesis or Mitosis as suggested by Hood et al. (1979) and Rang and Dale (1987) about the mitomycin-C activity.

**Cadmium**

Like other mutagens used in this study intraperitoneal injection of cadmium also led to the cell damage and nuclear anomaly. The frequency, of micronucleus, nuclear anomaly and nuclear lesion resulted in the animals exposed to various level of cadmium doses and for the different time periods, is given in table 10. A comparison between the nuclear lesion frequencies at each level of dose was made for each exposure time and is presented diagramatically in fig. 9.

It is clear from the table 10; that all treated animals showed higher incidences of nuclear lesions (MN+NA) than those of control animals. A linear increase in nuclear lesions was noted with the increase in time of exposure at 0.5 ppm of cadmium upto 72 hrs. whereas at other dose levels the trend continued upto 96 hrs. Similarly, for the same time of exposure there was an increase in the micronucleus, nuclear anomaly and thus nuclear lesions frequencies with the increase in dose (table 10).

Effect of dose and time was studied by conducting the two way ANOVA (table 11). Significant variation between doses (0, 0.5, 1.0 and 2.0 ppm) was observed ($P<0.01$) in micronucleus and nuclear anomaly induction. Significant variation ($P<0.01$) was also observed between time (24, 48, 72 and 96 hr) only in nuclear anomaly

Table 10: Frequency distribution (o/oo) of micronucleus, nuclear anomaly and nuclear lesion in animals exposed to cadmium.

| Treatment | Dose ppm | Frequency (o/oo) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hrs | | | 48 hrs | | | 72 hrs | | | 96 hrs | | |
| | | MN | NA | NL | MN | NA | NL | MN | NA | NL | MN | NA | NL |
| Control | - | 0.4 | 0.6 | 1.0 | 0.4 | 0.6 | 1.0 | 0.6 | 0.8 | 1.4 | 0.6 | 0.8 | 1.4 |
| Cadmium | 0.5 | 0.4 | 1.2 | 1.6 | 0.4 | 2.0 | 2.4 | 0.8 | 2.6 | 3.4 | 0.8 | 2.6 | 3.4 |
| | 1.0 | 1.4 | 2.0 | 3.4 | 1.6 | 3.2 | 4.8 | 2.2 | 3.8 | 6.0 | 4.0 | 4.4 | 8.4 |
| | 2.0 | 2.8 | 2.8 | 5.6 | 3.6 | 4.0 | 7.6 | 3.0 | 5.0 | 8.0 | 4.2 | 4.8 | 9.0 |

MN - Micronucleus, NA - Nuclear Anomaly, NL- Nuclear lesion

Table 11: Effect of Cadmium on micronucleus, nuclear anomaly and nuclear lesion.

| Source | D.F. | F-values | | |
|---|---|---|---|---|
| | | MN | NA | NL |
| Dose | 3 | 26.62** | 45.74** | 43.26** |
| Time | 3 | 3.32 | 9.57** | 6.70* |
| Error | 9 | | | |

** Significant at 1% level ($P<0.01$), * Significant at 5% level ($P<0.05$)

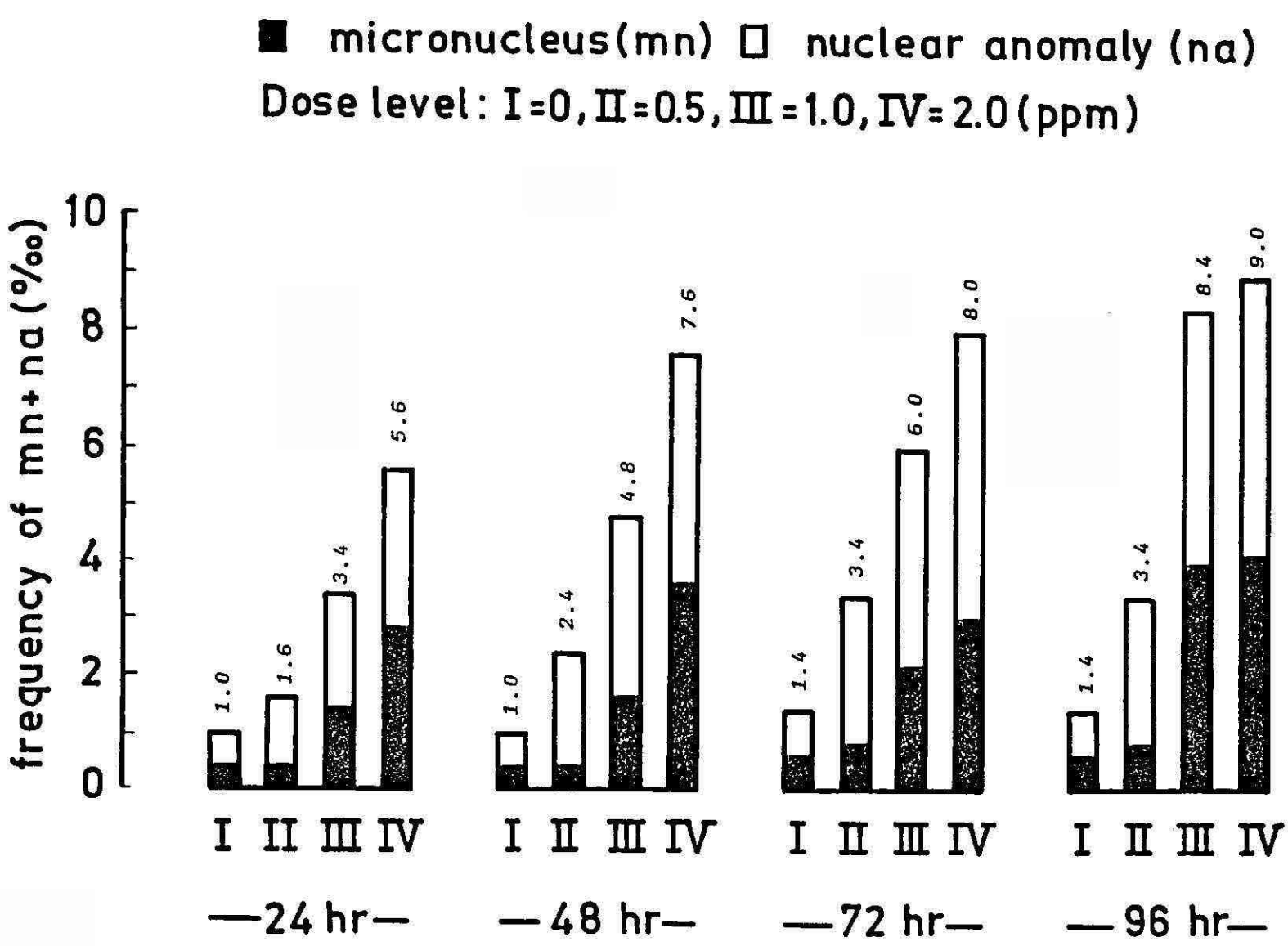



Fig. 9. TREATMENT WITH CADMIUM

formation, but not in micronucleus formation, where homogeneous effect of time was noted. C D analysis showed, the variation between the doses were in the order of 0 and 2.0, 0.5 and 2.0, 0 and 1.0 and 0.5 and 1.0 ppm for micronucleus production and 0 and 2.0, 0 and 1.0, 0.5 and 2.0, 0 and 0.5 and 0.5 and 1.0 ppm for nuclear anomaly incidence. The order for variation in time was 24 & 96 and 24 & 72 hrs for nuclear anomaly production. Rest all the combinations of time showed homogeneous effect. In total, the nuclear lesions production was significantly varying between doses ($P<0.01$) and time ($P<0.05$). The C D analysis for nuclear lesion showed the variation between the doses 0 and 2.0 ppm at maximum significant level, followed by 0.5 and 2.0, 0 and 1.0, 0.5 and 1.0 ppm similarly, maximum variation was observed between the time 24 and 96 hr. In rest of the combinations of time and dose the results were homogeneous in nature.

Increased micronucleus incidences after exposure to cadmium was in agreement with the reports of Manna and Sadhukhan (1986) and Gola et al. (1986) in tilapia and mussel respectively. Intraperitoneal injection of cadmium showed an elevated micronucleus than control as high as 127 micronuclei per thousand cells after 30 hrs of exposure in tilapia. Gola et al. (1986) observed similar increase in micronucleus production after 24 hrs of cadmium exposure to

mussels. Erythrocytic nuclear damages observed in the present study was similar to the report of Gill and Pant (1985) in Puntius conchonius.

In the present study, increased micronucleus and nuclear anomaly incidences after cadmium exposure might be due to the inability of the fish to detoxify the poison and the existing unexcreted toxic metabolites effect (Johnson - sjobeck and Larsson, 1978) leading to the loss in integrity of nuclear membrane and membrane bound enzymes by enhancing lipid peroxidation as suggested by Stacey and Kappus (1982 b) and Younes and Siegers (1984).

The time dependent nuclear anomalies might be due to the effect of cadmium on erythropoietic cells rather than on the circulating erythrocytes as described by Houston and Keen (1984) and Garofano and Hirshfield (1983).

Genotoxicity of all these chemicals might be a result of bio-activation, reaction with biomolecules, induction and persistence of micronucleus and nuclear anomaly. Although most of the genotoxic substances and their metabolites in general eventually reach many of the body cells; but haemotopoietic cells and peripheral blood cells because of their fast dividing nature are affected more. Thus the present increased micronucleus and nuclear anomaly incidences in erythrocytes might be purely due to genotoxicity of the test chemicals used. This is on the

basis of the basic principle that almost all the genotoxic agents are presumed to produce reactive metabolites and free radicals which may react with macromolecules to yield increased DNA damage through alkylation, cross-linking of DNA chain and interaction with membrane leading to increased micronucleus production.

In all the experiments, the frequency of micronuclei in control fish varied between 0.2 - 0.4 per thousand cells. This low value is in agreement with earlier reports made by Hooftman and de Raat (1982) Metcalfe (1988) Van Hummesen et al. (1989) and Williams and Metcalfe (1992) who have used piscine micronucleus test for chemical genotoxicity assessment.

The general dose and time dependent nuclear lesions induced by the chemicals tested agree with the principle that the clastogenic, mutagenic and other genotoxic agents cause chromosomal damage in the exposed cells and they can be visualized as micronuclei or nuclear anomalies. Chemical specific nuclear lesion incidences according to time and dose noted in the study were in agreement with the finding of Al-Sabti (1985a) who opined that the dose dependent increase of micronucleus incidences are pollutant specific.

The observation of nuclear anomalies in all the experiments agrees with Nikinmaa (1992), who have described the effect of chemicals on erythrocytes by impairing the physiological co-ordination in fish. The nuclear anomalies observed in the study coincides with the descriptions made in the earlier studies that used the piscine micronucleus test (Hooftman and de Raat, 1982; Longwell et al., 1983; Manna et al., 1985; Al-sabti, 1986 a, 1986 b; Das and Nanda 1986; Hose et al., 1987; Long and Buchman, 1989) and were not naturally produced as they were minimum in controls. However, it needs further study for confirmation as described by Carrasco et al. (1990).

The increased micronucleus incidences/total nuclear lesions observed in general in E. suratensis might be due to the reason that the activity of DNA polymerase, an enzyme important in DNA repair, is markedly less active in fish cells compared to rodent and human cells as reported by scovassi et al. (1979) and Walton et al. (1984). This DNA polymerase hypo-activity would have slowed down DNA repair which in turn caused more number of cells with DNA damage to undergo mitosis leading to more number of micronuclei and nuclear anomalies as suggested by Walton et al. (1984).

Almost in all the experiments, the micronucleus frequencies were low, but statistically significant. The

low frequency of micronuclei might be due to the inhibition of mitotic activity rather than a reduced level of genotoxic damage. The similar effect was also observed by Tates et al. (1983) and Das and Nanda (1986). However, it can be speculated that the relative low rate of erythropoiesis in fish species in general could also be the cause of low micronucleus frequencies. Metcalfe (1988) suggested that the low frequency of micronuclei noted in fish in vivo may not be due to any cellular resistance to clastogenic activity.

The results of this study indicate that the three chemical/mutagens and one heavy metal selected are genotoxic to E. suratensis. Incidence of micronuclei reported in mammals (Heddle et al., 1983; Sutou et al., 1986; Gupta, 1989) had similarities with the present investigation. This is in the agreement with the basic principle that an agent which is genotoxic for one group of living organisms is also genotoxic for other, because of the universality of DNA as stated by Landolt and Kocan (1983).

Further, it is supported by the report of Lech and Bend (1980), Binder et al. (1984) and Hunn and Greer (1991) that the fish mixed function oxidase (MFO) system is almost similar to mammals. Hence it can substitute or supplement costly mammalian models for the evaluation of toxic effects

of chemicals in human system or to conduct pre-marketing survey of commercial chemicals, which have adverse effects on biota. This concept of fish as a 'model patient' in toxic studies was also recommended by Powers (1989) and other researchers (Metcalfe, 1989; Metcalfe et al., 1990; Thomas, 1990; Nishimoto et al., 1991; Zhang et al., 1992; Bailey et al., 1992; Reichert et al., 1992; Schnitz and O' connor, 1992; Kubota et al., 1992; Adams et al., 1992; Marafante and Clerici, 1992; Aoki et al., 1993) who have studied the genetic responses of toxicity in fish.

Moreover, the present study indicated the rapidity and simplicity of piscine erythrocytic micronucleus test over any other test system. Due to the nucleated erythrocytes in fish, the observation and characterisation of micronucleus/nuclear anomaly in such cells was easy and had the advantage of reducing the artifacts by direct comparison of micronucleus with main nucleus, which is not possible in Mammalian micronucleus test. Where the erythrocytes are enucleated. This is in agreement with the statement of Hooftman and de Raat (1982) that the cells with well defined nucleus and prominent boundary facilitates the identification of fragments in their cytoplasm.

## Field Study

The field study was conducted by analysing the natural incidence of micronucleus, nuclear anomaly and

nuclear lesion in the fishes occuring in suspected polluted sites (Eloor industrial area on the bank of Periyar and Narakkal waste water canal, Kochi). The frequency(0/00) of nuclear abnormalities in the form of micronucleus, nuclear anomalies and nuclear lesion, occurring in these animals have been given in table 12. Animals collected from Matsyafed fish pond Narakkal, which is considered to be a pollution free pond, were used as control animals. A comparison between nuclear lesions of treated animals and field samples, has been depicted in Fig. 10.

In Eloor industrial area, frequency (0/00) of micronuclei and nuclear anomalies varied between 6.8 - 8.2 and 11.4 - 14.4 respectively whereas the total nuclear lesions varied between 18.2 - 22.2. In the animals collected from Narakkal waste water canal, micronucleus and nuclear anomaly incidences (0/00) varied between 2.0 - 2.6 and 5.4 to 6.2 respectively against the range of 0.1 - 0.2 and 0.4 - 0.7 in control animals (table 12). In Eloor industrial area and Narakkal waste water canal, the nuclear lesions showed a 25 and 11 times increase than control incidences respectively.

During all the sampling periods, nuclear lesions were found to be very high in animals occuring in Eloor industrial area followed by Narakkal waste water canal against a low incidence in control station.

Table 12: Micronucleus (MN) and Nuclear Anomaly (NA) incidences in suspected polluted areas and control stations

| Sampling Period | Matsyafed fish pond, Narakkal | | | Narakkal waste water canal | | | Eloor Industrial area | | |
|---|---|---|---|---|---|---|---|---|---|
| | MN (o/oo) | NA (o/oo) | Total (o/oo) | MN (o/oo) | NA (o/oo) | Total (o/oo) | MN (o/oo) | NA (o/oo) | Total (o/oo) |
| September '93 (IV week) | 0.2 | 0.7 | 0.9 | 2.6 | 6.0 | 8.6 | 7.8 | 14.4 | 22.2 |
| October '93 (II week) | 0.2 | 0.5 | 0.7 | 2.0 | 5.4 | 7.4 | 8.2 | 13.6 | 21.8 |
| October '93 (IV week) | 0.1 | 0.8 | 0.9 | 3.0 | 6.2 | 9.2 | 6.8 | 11.4 | 18.2 |

MN- Micronucleus, NA- Nuclear Anomaly.

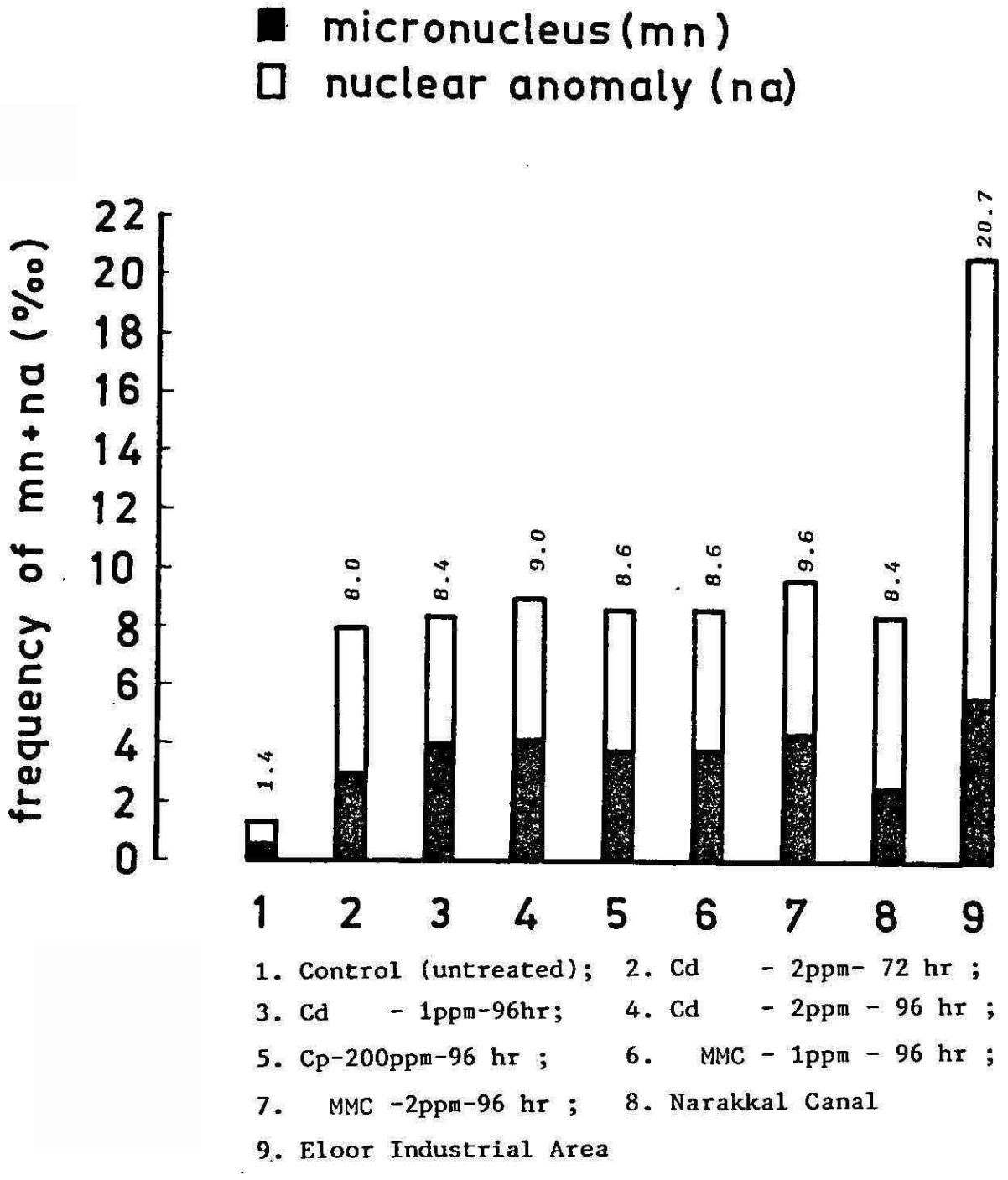



Fig.10. Comparision of Treated & Field Samples

Eloor industrial area is reported to be highly polluted with a variety of chemicals including cadmium (Gopalan, 1990 ; Joy, 1990). This high range of pollutants including cadmium might have caused the increased incidence of micronucleus and nuclear anomalies observed in this study. Narakkal waste water canal is inferred to be contaminated with house-hold and other inorganic and organic wastes. Fishes sampled from this area also showed significant estimates of micronucleus than control animals.

In a similar study, high incidences of micronucleus/ nuclear anomaly were also observed by Hose et al. (1987) in California coast contaminated with chlorinated hydrocarbons. Four to eleven fold increase in micronucleus frequencies than reference site were observed by them. Similarly significant correlation between contamination (with urban discharge & industrial discharge) and micronucleus incidences were noted by Brunetti et al. (1988) in Mytilus galloprovincials (mussel). Cross et al. (1987) also observed high level of micronuclei in kelp bass larvae residing in an urban bay rather than in rural bay. The higher incidences of micronucleus/nuclear anomaly in animals inhabiting polluted habitats identified and sampled in the study, when compared with experimental results (Fig. 10) brings forth the severity of contamination in the

sampled locations. In the laboratory animals exposed to cadmium showed elevated micronuclei/nuclear anomalies than control. From this it can be inferred that the elevated micronuclei/nuclear anomalies observed in Eloor industrial area might have resulted from the effect of cadmium or from the synergistic effect of cadmium with other contaminents present in that area. However it needs further confirmation.

The present study suggested the possibilities of using the frequencies of micronucleus measured in natural populations and in chemically exposed populations of aquatic organisms, as a rapid and reliable test to monitor the aquatic environment for the presence of genotoxic pollutants and to evaluate the potency of chemicals for the genotoxicity at sublethal level.

Moreover, the micronucleus test bypasses all the problems posed by other cytogenetic techniques such as the chromosome aberrations and the sister chromatid exchange tests. Owing to the advantages of micronucleus test over the other techiniques and ease to perform this test can be used as a suitable method for in situ monitoring of feral fish for genotoxicity. However, further investigations to decipher the influences of various biotic and abiotic parameters may add to the suitability of this test.

# SUMMARY

# SUMMARY


1. The study was conducted in Etroplus suratensis using Micronucleus Test (MNT) with the objectives (i) to detect various types of nuclear anomalies of fish erythrocytes, (ii) to detect artifacts seen in piscine erythrocytic micronucleus test (iii) to generate base-line data to screen known chemicals for genotoxic potential and (iv) to assess genotoxic effects in fish from suspected polluted sites with a standardised protocol for piscine erythrocytic micronucleus test.

2. Among Sodium citrate (2%) and Heparin (4 mg/ml). The later was found to be a suitable anticoagulant which prevented the blood clotting effectively and did not damage the erythrocytes.

3. Different procedures were tried to get an optimum method for blood slide preparation and staining. Giemsa stain (20%) made in Sorensen's buffer (pH 6.8) was found to be the best for Piscine erythrocytic micronucleus test (MNT).

4. Low counts of micronuclei (MN) and nuclear abnormalities (NA) were found in the animals collected from Matsyafed fish pond and CIBA fish pond, Narakkal.

The counts ranged from 0.0-0.3 o/oo for micronuclei and 0.2-0.6 for nuclear anomalies. Which provided the base – line frequencies (background counts) for the studies conducted in the laboratory animals treated with known chemical mutagens and collected from the suspected polluted areas.

5. Many artifacts in the form of overlapping cells, and ruptured cells were observed alongwith micronuclei and nuclear anomalies. Stain particles and acidic materials other than chromosomal fragments were the main intervening artifacts.

6. In the laboratory; the animals were treated with different doses of colchicine (0.1, 0.5 and 1.0 ppm), cyclophosphamide ( 100, 150 and 200 ppm), mitomycin-C (0.5, 1.0 and 2.0 ppm) and cadmium (0.5, 1.0 and 2.0 ppm). The blood samples were collected from these animals after 24, 48, 72 and 96 hrs. and were subjected micronucleus test.

7. In animals treated with colchicine, maximum frequency (0/00) of micronuclei and nuclear anomalies ranged from 0.6 to 3.2 and 1.4 to 3.6 respectively at 1.0 ppm after 96 hrs. Level of dose showed significant effect ($P < 0.01$) on

micronucleus, nuclear anomaly and total nuclear lesions ( i.e. MN+NA) incidence. Whereas time had significant effect ($P<0.05$) on nuclear anomalies and nuclear lesions but not on micronuclei.

8. In animals treated with alkylating agent, cyclophosphamide, higher incidence of micronuclei and nuclear anomalies was observed at 200 ppm after 96 hrs. Total nuclear lesions (MN + NA) ranged from 5.4 to 8.6 o/oo. Both dose and time had significant effect ($P<0.01$) on the micronuclei and nuclear anomalies production.

9. In animals treated with mitomycin-C (aneuploidogenic agent), maximum micronuclei and nuclear anomalies incidence was observed at 2.0 ppm dose after 96 hrs of the treatment. The total nuclear lesions ranged from 4.8 to 9.6 o/oo whereas after the same time period it was 0.8 in control animals. Variation between doses (0, 0.5, 1.0 and 2.0 ppm) and time (24, 48, 72 and 96 hrs) was significant ($P<0.01$) in micronuclei and nuclear anomalies production.

10 Like other chemicals cadmium also induced the elevated micronucleus and nuclear anomaly in treated animals against a low incidence of control. An increasing trend in micronuclei, nuclear anomalies and nuclear lesions was observed with

increase in dose and time. Significant variation ($P<0.01$) between doses was observed in micronucleus and nuclear anomaly induction. Significant variation between time of exposure was observed for nuclear anomalies but not micronuclei.

11. Field study was conducted by analysing the natural incidence of micronucleus, nuclear anomaly and nuclear lesion in the animals occurring in suspected polluted areas (Eloor industrial area of river Periyar and Narakkal waste water canal, Kochi). The micronuclei and nuclear anomalies varied between 6.8-8.2 and 11.4-14.4 o/oo respectively in animals from Eloor industrial area. Whereas in animals from Narakkal waste water canal micronuclei and nuclear anomalies ranged between 2.0-2.6 and 5.4-6.2 o/oo respectively. The nuclear lesions in Eloor industrial area and Narakkal waste water canal respectively showed a 25 and 11 times increase than the control incidence.

# CONCLUSION AND RECOMMENDATIONS

## CONCLUSION & RECOMMENDATIONS

The study concluded that Etroplus suratensis can be used as a good model to study the genotoxic effects of aquatic Pollutants in fishes. The high incidence of micronuclei, nuclear anomalies and nuclear lesions in animals from polluted areas showed that the aquatic animals in these areas are under serious threat of pollution.

Due to the nucleated erythrocytes in fish, the piscine erythrocytic micronucleus test can be used as a tool to monitor the aquatic pollution and to evaluate the genotoxic potency of commercial chemicals. Moreover, the simplicity, rapidity and accuracy of this test add to its suitability for genotoxic studies.

Since fish is an important part of our food it is recommended to undertake a large - scale genotoxicity screening of chemicals, effluents and environmental residues, etc. to check the level of contamination in fishes of economic importance where E. suratensis can be used as a cytogenetic model and piscine erythrocytic micronucleus test as a cytogenetic tool and thereby formulate the pollution control programmes accordingly.

# BIBLIOGRAPHY

## BIBLIOGRAPHY


ADAMS, S.M., W.D.CRUMBY, M.S.GREELEY JR., L.R.SHUGART AND C.F.SAYLOR, 1992. Responses of fish populations and communities to pulp mill effluents: a holistic assessment. Ecotoxicol. Environ. Safety, 24(3): 347-360.

ALINK, G.M., FEDERIC-WALTERS AND E.M.H.VAND, 1980. Induction of sister chromatid exchanges in fish-exposed to Rhine Europe water. Mutation Res., 78: 369.

AMES, B.N. 1971. The detection of chemical mutagens with enteric bacteria. In: chemical mutagens, principles and methods for their detection. Hollaender, A. (Ed.), NY. 1: 267-287.

AOKI, K., Y.NAKATSURU, J.SAKURAI, A.SATO, P.MASAHITO and T.ISHIKAWA, 1993. Age dependence of $O_6$-methylguanine DNA methyltransferase activity and its depletion after carcinogen treatment in the teleost medaka (Oryzias latipes). Mutation Res., 293(3): 225-231.

AL-SABTI, KABIL. 1985 a. Carcinogenic-Mutagenic chemicals induced chromosome aberration in the Kidney cIles of three cyprinds. Comp. Biochem. Physiol.C, 82(2):484-493.

AL-SABTI, KABIL. 1985 b. Frequency of chromosomal aberration in the rainbow trout (S.gairdneri Richardson) exposed to 6 pollutants. J. Fish Biol., 26(1): 13-19.

AL-SABTI, KABIL. 1986 a. Comparative micronucleated erythrocyte cell induction in 3 cyprinds by 5 carcinogenic-mutagenic chemicals. Cytobios, 47 (1990/(91): 147-154.

AL-SABTI, KABIL. 1986 b. Clastogenic effects of 5 carcinogenic Mutagenic chemicals on the cells of the common Carp. Cyprinus carpio L. Comp. Biochem. Physiol., 85(1):5-9.

ATHWAL, R.S. and S.S.SANDHU, 1985. Measurement of chemically induced mitotic non-disjunction using a monochromosomal Human/Mouse hybrid cell line. In: short-term Bioassays in the analysis of complex Environmental mixtures IV, Waters, M.D., S.S.Sandhu, J.Lowtas, L.Claxton, G.Staruss and S.Nesnon, (Ed. ), Plenum press, NY. 99-112.

AUERBACH. C. and J.M.ROBSON, 1946. Chemical production of Mutations. Nature, 157-302.

BAKSI, S.M. and J.C.MEANS, 1989. Development of a mutagenicity assay on early life stages of Morone saxitilis and Cyprinodon varigatus. Environ. Mutagen.

BAKS I, S.M. and J.M.FRAZIER, 1990. Raview:Isolated fish hepatocytes-model systems for toxicology research. Aquat. Toxicol., 16: 229-256.

BAILEY, G., J.HENDRICKS and R.DASHWOOD, 1992. Anticarcinogenesis in fish. Mutation Res., 267 (2): 243-250.

BINDER, R.L., M.J.MELANOCON and J.J.LECH, 1984. Factors influencing the persistence and metabolism of chemicals in fish. Drug Metal. Rev., 697-724.

BARKER,C.J. and B.D.RACKHAM, 1979. The induction of sister chromatid exchanges in cultured fish cells (Ameca splendens) by Carciogenic mutagens. Mutation Res., 68: 381.

BEARDMORE, J.A., C.J.BARKER, B.BATTAGLIA, R.J.BERRY, A.G.LONGWELL. J.F.PAYNE and A.ROSENFIELD, 1980. The use of Genetical approaches to monitoring Biological effects of pollution. Rapp. P.-V.Reun. Cons. Int. Explor. Mer., 179:

BERRY, R.J. 1980. Gens, pollution and monitoring. Rapp. P.-V. Reun. Cons. int. Explor. Mer., 179: 253-257.

BHAUMIK (ROY), S., D.P.MUKHERJEE and R.SINHA, 1987. Species and sex Differences in the dimensional characteristics of Erythrocytes of fish. J.Inland Fish. Soc. India, 1: 1-8.

BOUDOU, A., D.GEORGESCAULD and J.P.DESMAZES, 1983. Ecotoxicological role of the membrane barriers in transport and bioaccumulation of Mercury compounds. In. Aquatic toxicology, Vol. 13, Nriagu, J.O. (Ed.), John Wiley and Sons, NY. 117-136.

BROOKES, P. and P.D. LAWLEY, 1971. In: chemical Mutagens, Principles and methods for their detection; Hollaender, A (Ed.), Plenum press, NY. 1:P.121.

BRUSICK, D.J. 1980. Principles of genetic toxicology (2nd Edn.), Plenum Press, NY. P. 279.

BRUNETTI, R., F.MAJONE, I.GOLA and C.BELTRAME, 1988. The micronucleus test: examples of application to marine ecology. Mar. Ecol. Prog. Ser., 44: 65-68.

BRUNETTI, R., O.FUMAGALLI, P.VALERIO and M.GABRIELE, 1992. Genotoxic effects of anoxia on Mytilus galloprovincialis. Mar. Ecol. Prog. Ser., 83: 71-74.

BRUNETTI, R., M.GABRIELE, P.VALERIO and O.FUMAGALLI, 1992. The micronucleus test: temporal pattern of base line frequency in Mytilus galloprovincialis. Mar. Ecol. Prog. Ser., 83: 75-78.

BUHLER, D.R. and D.E.WILLIAMS, 1988. The role of biotransformation in the toxicity of chemicals. Aquat. toxicol., 11: 19-28.

CAPUZZO, J.M., M.N.MOORE and J.WIDDOWS, 1988. Effects of toxic chemicals in the marine environment. Predictions of impacts from laboratory studies. Aquat. toxicol., 11: 303-311.

CARRASCO, K.R., K.N.TILBURY and M.S.Mysers, 1990. Assessment of Piscine MicronucleusTest as an in situ Biological indicator of chemical contaminant effects. Can. J. Fish. Aquat. Sci., 47: 2123-2136.

CROSS, J.N., J.T.HARDY, J.E.HOSE, G.P.HERSHELMAN, L.D.ANTRIM, R.W.GOSSETT and E.A.CRECELIUS, 1987. Contaminant concentrations and toxicity of sea surface Microlayer near Los Angles, California. Mar. Environ. Res., 23: 307-327.

DANIEL, J.W. and L.GOLBERG, 1980. Round Table, survey on current methods and new approaches to the Evaluation of Mutagenic potential. In: The principles and methods in modern toxicology, Galli, C.L., S.D. Murphy and R.Paoletti (Eds.), Elsevier/North-Holland Biomedical press. 141-166.

DANIELS, C.B., I.CHANDLER and T.CHEN, 1992. Assessment of the cytogenicity of a Brass effluent in juvenile Cyprinodon varigatus. Environ. Mol. Mutagenes. 19(20): 12.

DAS, R.K. and N.K.Nanda, 1986. Induction of micronuclei in peripheral erythrocytes of fish Heteropneustes fossilis by mitomycin-c and paper mill effluent.Mutation Res., 175: 67-71.

DASS, P. 1990. Genetic response to toxicity in Etroplus suratensis (Bloch) and E.maculatus (Bloch) Ph.d thesis, submitted to Cochin University of Science and Technology, Cochin.

* DE VRIES, H. 1901-1903. Die Mutation Theorie. Veit Leepzig. (The Mutation Theory, Tr. 1909 Open Court, Chicago).

EPA. 1984. Research and development. Index and Abstracts to publications. The EPA-NCI project. Effects of Carcinogens, Mutagens and Teratogens on non-Human species (Aquatic animals). Environmental Research laboratory, Gulf Breeze, Florida (USA). P.91.

ESTMOND, A.D. and J.D.TUCKER, 1989. Kinetochore localisation in Micronucleated cytokinesis-blocked chinese hamster ovary cells: a new and rapid assay for identifying aneuploidy-inducing agents. Mutation Res., 224: 517-525.

EVANS, H.J., G.J.NEARY and F.S.WILLIAMSON, 1959. The relative biological efficiency of single doses of fast neutrons and gamma rays in vicia faba roots and the effects of oxygen. II. Chromosome damage: the production of micronuclei. Intern. J. Radiation Biol., I, 216-229.

EVANS, E.J. 1976. Cytological Methods for detecting chemical Mutagens. In: Chemical mutagens, Principles and methods for their detection, Vol.4. Hollaender, A.(Ed.), Plenum press, NY. 1-29.

FLOWER, R.J., S.MONCADA and J.R.VANE, 1980. Analgesic antipyretics and anti-inflammatory agents: drugs employed in the treatment of Gout. In: Goodman and Gilman's, The Pharmacological basis of Therapeutics (6th Edn.), Gilman, A.G., L.S.Goodman, A.Gilman, S.E.Mayer and K.L.Melmon (Eds.) Mac Millan Publishing Co., Inc., NY. 718-728.

FOLMAR, L.C., T.MOODY, S.BONOMELLI and J.GIBSON, 1992. Annual cycle of blood chemistry parameters in striped mullet (Mugil cephalus) and pinfish (Lagodon rhomboides) from the Gulf of Mexico.J. Fish Biol., 41: 999-1011.

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* Not referred in original.

GARDENER, G.R. and P.P.YEVICH, 1970. Histological and hematological responses of an estuarine teleost to cadmium. J. Fish Res. Board Can., 27: 2185-2196.

GABRIELE, M., R.BRUNETTI and F.MAJORE, 1992. The Micronucleus Test in cultured Mammalian cells and in Mytilus gill tissues Italian Tissue culture Meeting, ATLA, 20: 103-107.

GAROFANO, J.S. and H.I.HIRSHFIELD, 1983. Peripheral effects of cadmium on the blood and head kidney of the brown bullhead (Ictalurus nebulosus). Bull Environ. Contam. Toxicol., 28: 552-556.

GESAMP, 1991. (IMO/FAO/Unesco/WMO|WHO/IAEA/UN/UNEP Joint group of experts on the Scientific Aspects of Marine Pollution). review of potentially harmful subtances-carcinogens, Rep. stud. GESAMP, (46), 56 pp.

GILL, T.S. and J.C.PANT, 1985. Erythrocytic and Leukocytic responses tc cadmium and poisoning in a freshwater fish, Puntius conchonius (Ham) Environmental Research, 36; 327-337.

GOODIER, J., HAI-FEIMA and FUMIO YAMAZAKI, 1987. Chromosome fragmentation and loss in two salmonid Hybrids. Bulletin Fac. Fish. Hokkairo Univ., 38: 181-184.

GOPALAN, U.K. 1992. Periyar-the life line of Kerala. In: Environmental Hazards in Kerala. Problems and remedies: Environment Monitoring Forum, Kochi, Kerala, Pauthenthara et al. (Eds.). 21-27.

GOLA, I.R., R.BRUNETTI, F.MAJONE and A.G.LEVIS 1986. Applications of the Micronucleus test to a Marine organism treated with NTA insoluble heavy metals. Atti Associazione Genetica Italiana, XXXII: 95-96.

GRINFIELD, S., A.JAYLET, R.SIBOULET, P.DEPARIS and I. CHOUROULINKOV, 1986. Micronuclei in red blood cells of the newt Pleurodeles waltl after treatment with benzo (a) pyrene: dependence on dose, length of exposure, post treatment time, and uptake of the drug. Environ. Mutagen. 8: 41-51.

GROLIMAN, A. 1960. Antipyretic and antirheumatic analysis. In: Pharmacology and Therapeutics. Lea and Febiger, Philiadelphia. 150-173.

**GUPTA, I.D. 1989.** Genotoxic effects of pesticidal drugs in sheep and laboratory animals. Ph.D thesis submitted to the Kurukshetra University, Kurukshetra, NDRI, Karnal, India.

**HAMILTON, R.D. 1976.** Aquatic environmental quality: toxicology. J. Fish. Res. Board. Can., 33: 2671-2688.

**HARRISON, F.L. 1987.** Effects of radiation on frequency of chromosomal aberrations and SCE in Neanthes arenaceodenta. In: Oceani process in marine pollution-1. Biological Processes and wastes in the ocean. Capuzz, J.M. et. al. (Eds.), Fl(USA): 145-156.

**HAYASHI, M., T.SOFUNI, and M.ISHIDATE. Jr. 1984.** Kinetics of micronucleus formation in relation to chromosomal aberrations in mouse bone marrow. Mutation Res., 127: 129-137.

**HEDDLE, J.A. 1973.** A rapid in vivo Test for chromosomal damage. Mutation Res., 18: 187-190.

**HEDDLE, J.A., H.MARK, B.KIRKHART, K.MAVOURNIN, J.T.MAC GREGOR, G.W.NEWELL and M.F.SALAMONE, 1983.** The induction of micronuclei as a measure of genotoxocity. A report of the U.S.Environmental protection Agency Gene-Tox Program. Mutation Res., 123: 61-118.

**HOEVEN, VAN DER J.C.M., I.M.BRUGGEMAN, G.M.ALINK and J.M.KOEMAN, 1982.** The Killifish Nothobranchius rachowi, a new animal in genetic toxicology. Mutation Res., 97: 35-42.

**HOOD, L.E., I.L.WEISMAN and W.B.WOOD, 1979.** Immunology, The Benjamin/cummings publishing company, Inc. London. 89.

**HOOFTMAN, R.N. 1981.** The induction of chromosome aberration in Notobranchius rochowi after treatment with ethyl methanesulphonate or Benzo (a) pyrene. Mutation Res., 91: 3474-352.

**HOOFTMAN, R.N. and G.VINK, 1981.** Cytogenetic effects on the eastern mudmirrow, Umbra pygmaea exposed to Ethylmethane-sulphonate (Ems), Benzo (a) Pyrene and River water. Ecotoxical. Environ. Safety, 5: 261-269.

**HOOFTMAN, R. N. and W.K.DE RAAT, 1982.** Induction of nuclear Anomalies (Micronuclei) in the Peripheral blood erythrocytes of the eastern mudminnow Umbra pygmaea by ethylmethane sulphonate. Mutation Res., 014 (147): 147-152.

HOSE, J.E., J.N.CROSS, S.G.SMITH and D.DIEHL, 1987. Elevated circulating erythrocytic Micronuclei in fishes, from contaminated sites of off southern California. Marine Environmental Research, 22: 167-176.

HOUSTON, A.H. and J.E. KEEN, 1984. Cadmium inhibition of erythropoiesis in gold fish, Carassius auratus. Can. J. Fish. Aquat. Sci., 14: 1829-1834.

HOWELL, W.H. 1891. The life-history of the formed elements of the blood, especially of the red blood corpuscles. J. Morphol., 4: 57-116.

HUNN, J.B. and I.E. GREER, 1991. Influence of sampling on the blood chemistry of Atlantic salmon. Progr. Fish culturist, 53: (3):184-187.

JAYLET, A., P.DEPARIO and D.GASCHIGNARD, 1986. Induction of Micronuclei in Peripheral erythrocyte of axolotl larvae following in vivo exposure to Mutagenic agents. Mutagenesis, 1(3).

JAYLET, A., L. GAUTHIER and C.ZOLL, 1990. Micronucleus test using peripheral red blood cells of amphibian larvae for detection of genotoxic agents in fresh water pollution. In: In situ evaluation of biological hazards of environmental pollution, Sandhu, S.S., W.R.Lower, F.J. de Serres, W.A. Suk and R.R. Tice (Eds.), Plenum press, NY. P.71.

JENSSEN, D. and C.RAMEL, 1980. The micronucleus test as part of a short term mutagenicity test programme for the prediction of carcinogenicity evaluated by 143 agents tested. Mutation Res., 75, 191-902.

JOHNSSON-SJOBECK, M-L. and A.LARSSON, 1978. The effect of cadmium on the hematology and on the activity of aminolevulinic acid dehydrate (ALA-D) in blood hematopoietic tissues of flounder, Pleuronectus flesus L. Environ. Res., 191-204.

* JOLLY, J. 1905. Sur 1' evolution des globules rouges dans le sans des embryons de mammiferes. C.R.Seans. Soc. Biol. (Paris), 58: 5933-595.

JOY, C.M. 1992. River Periyar and pollution problems. In: Environmental Hazards in Kerala. Problems and remedies, Environmental monitoring Forum, Kochi, Pauthenthara, et al. (Eds.). 29-36.

JONES, J. NIGEL and J.M. PARRY, (1992). The detection of DNA adducts, DNA base Changes and chromosome damage for the assessment of exposure to genotoxic pollutants. Aquat. toxicol., 22 (4): 323-344.

KATO, H. and H.SHIMADA, 1975. Sister chromatid exchange induced by Mitomycin: A new method of detecting DNA damage at chromosomal level. Mutation Res., 28: 459-464.

KATZ, M. 1950. Some increasing cells in the Blood of a diseased Silver Salmon Fingerling. Copea, 4: 293-298.

KERKHOFF, J.F.J. and M.A. GAAG, 1985. Some factors affecting optimal differential staining of sister-chromatids in vivo in the fish Nothobranchius rachowi. Mutation Res., 143: 39-43.

KIRKHART, B. 1981. Micronucleus Test on 21 compounds. In: Evaluation of short-term tests for carcinogens. report of the International collaborative program, de serres, F.J. and J.Ashby (Eds.), Progress in mutation Research Vol.1, Elsevier/North-Holland, NY. 698-704.

KLAASSEN, C.D. 1980. Heavy metals and Heavy metal antagonists. In: Goodman and Gilmans. The Pharmacological basis of Therapeutics (6th Edn.), Gilman, A.G., L.S. Goodman, A.Gilman, S.E.Mayer and K.L.Melmon (Eds.), Mac Millan Publishing Co., Inc. NY 1615-1637.

KLIGERMAN, A.D., S.E.BLOOM and W.M.HOWELL, 1975. Umbra limi: A model for the study of chromosome aberration in fishes. Mutation Res. 31: 225-233.

KLIGERMAN, A.D. and S.E.BLOOM, 1976. Sister chromatid differentiation and exchange in adult mud minnows (Umbra limi) after in vivo exposure to Bromodeoxyuridine. chromosome (Berl), 56: 101-109.

KLIGERMAN, A.D. 1979 c. Induction of sister chromatid exchanges in the central mudminnow following in vivo exposure to mutagenic agents. Mutation Res. 64: 205-217.

KLIGERMAN, A.D., J.WILMER and G.EREXSON, 1981. Characterisation of a rat lymphocyte culture system for assessing sister chromatid exchange after in vivo exposure to xenotoxic agents. Environmental mutagenesis, 3: 531-543.

KOCAN, R.M., M.L.LANDOLT and K.M.SABO, 1982. Anaphase aberration. A measure of genotoxicity in mutagen treated fish cells. Environmental Mutagen., 4: 181-189.

KRISHNAJA, A.P. and M.S.REGE, 1982. Induction of chromosomal aberration in fish Boleopthalmus dussumeri, after exposure in vitro to mitomycin-c and heavy metals, mercury, selenium and chromium. Mutation Res., 102: 71-82.

KUBOTA, Y., A.SHIMADA and A.SHIMA, 1992. Detection of gamma-ray induced DNA damages in malformed dominant lethal embryos of the Japanese medaka (Oryzias latipes) using AP-PCR finger printing. Mutation Res., 283(4): 263-270.

LANDOLT, M.L. and R.M.KOCAN, 1983. Fish cell cytogenetics: A measure of Genotoxic effects of Environmental pollutants. In: Aquatic toxicology, Vol. 13. Nriagu, J.D.(Ed.), John wiley and sons, N.Y. 335-353.

LARSSON, A. 1975. Some biochemical effects of cadmium on fish. In: "Sublethal Effects of Toxic chemicals on aquatic Animals", Koeman, K.H. and J.J.T.W.A.Strik, (Eds.), Elsevier, Amsterdam, 3-13.

LATT, S.A. 1974a. Sister chromatid exchanges, indices of human chromosome damage and repair: detection by fluorescence and induction by mitomycin-c. Proc. Nat. Acad. Sci. (USA), 71: 3162-3166.

LATT, S.A. 1974 b. Localization of sister chromatid exchanges in human chromosomes. Science, 185: 74-76.

LAPRIENO, N. 1980. General principles of Genetic toxicology and methods for Mutagenesis Assessment. In: The principles and methods in modern toxicology, Galli, C.L., S.D. Murphy and R.Paoletti (Eds.), Elsevier/North-Holland Biomedical press,. 107-124.

LECH, J.J. and J.R. Bend, 1980. Relationships between biotransformation and toxicity and fate of xenobiotic chemicals in fish. Environ. Health prospect., 34:115-131.

LIU, T.Y., S.L.CHENG, T.H.UENG, Y.F.VENG, C.W.CHI, 1991. Comparative analysis of aromatic DNA adducts in fish from polluted and unpolluted areas by the 32 p- postlabelling analysis. Bull. Environ. Contam. Toxicol., 47(5): 783-789.

LLOYD, R. 1980. Toxicity testing with aquatic organisms. A framework for hazard assessment and pollution control. Rapp. P-V. Reun. Cons. int. Explor. Mer., 179: 7-15.

LONG, E.R. and M.F.BUCHMAN, 1989. An evaluation of candidate measures of biological effects for the National status and Trends programme Nat'l oceanic Atoms. Adm. Tech.Memo. NOS OMA-45: 97 p.

LONGWELL, A.C., D.Perry, J.B.HUGHES and A. HEBERT, 1983. Frequencies of micronuclei in mature and immature lymphocytes of fish as an estimate of chromosome mutation rates results of field surveys on windowpane flounder, winter flounder and Atlantic mackerel. Proceedings of Council Meeting of the International Council for the exploration of Sea (Sweden): 14 (7).

LUCKY, M.V.Dr.ZDENEK. 1977. Haematological investigation of fish. In: Methods for the Diagnosis of fish Diseases, Hoffman, G.L. (Ed.), Amerind Publishing Co.Pvt. Ltd. NY. 127-137.

MAC GREGOR, J.T., C.M.WEHR and D.H.GOULD, 1980. Clastogen induced micronuclei in peripheral blood erythrocytes: of the basis of an improved micronuclei test. Environmental Mutagenesis, 2(4) : 509-514.

MAJONE, F., R.BRUNETTI, I.GOLA and A.G.LEVIS, 1987. Persistence of Micronuclei in the marine mussel, Mytilus galloprovincialis after treatment with mitomycin-C. Mutation Res., 191: 157-161.

MAJONE, F., C.BELTRAME and R.BRUNETTI, 1988. Frequencies of micronuclei detected on Mytilus galloprovincialis by different staining techniques after treatment with Zinc chloride. Mutation Res., 209: 131-134.

MAJONE, F., R.BRUNETTI, O.FUMAGLLI, M.GABRIELE and A.G.LEVIS, 1990. Induction of micronuclei by Mitomycin-C and colchicine in the marine mussel, Mytilus galloprovincialis. Mutation Res., 244: 147-151.

MAHAJAN, C.L. and J.M.S. DHEER, 1980. An antoradiographic and cytochemical study of erythropoiesis in a fresh water fish, Channa punctatus (Bloch.) J. Fish Biol., 17: 641-648.

MANNA, G.K. 1982. The new Horizon of Mutagenesis. Current Science, 51: 1087-1093.

MANNA, G.K., G.BANERJEE and S.GUPTA, 1985. Micronucleus test in the peripheral erythrocytes of the Exotic fish, Oreochromis mossambica. Nucleus (Calcutta), 28: 176-179.

MANNA, G.K. 1986. Tilapia as a model for testing genotoxic agents. In: Perspectives in Cytology and Genetics. 395-406.

MANNA, G.K. and A.SADHUKHAN, 1986. Use of cells of gill and Kidney of Tilapia fish in Micronucleus test (MN). Current Science, 55: 498-501.

MANNA, G.K. 1989. Fish cytogenetics related to taxonomy, evolution and Monitoring aquatic genotoxic agents. In: Fish Genetics in India, Das, P., and A.G. Ghingran (Eds.), Today and Tommorrows printers and publishers, New Delhi. 21-46.

MARAFANTE, E. and L.CLERICI, 1992. Biomarkers of fish Exposure to chemical Mutagens in the Assessment of water quality. Mutation Res., 27(2): 186-187.

MARSHALL, E.1993. Toxicology goes Molecular. Science, 259: 1394-1398.

MEANS, J.C., C.B.DANIELS and S.M.BAKSI, 1988. development of in vivo Genotoxocity tests in Estuarine fish and their Application to Aquatic Toxicology. Mar. Environ. Res., 24: 327-331.

MEIER, J., M.ROBINSON, R.DOBBS, M.JELUS, K.JENSEN, J. TIETGE and R.JOHNSON, 1992. Bioassay-directed fractination of a Coke oven waste water for Application to tumorigenicity testing in medaka (Oryzias latipes). Environ. Mol. Mutagen., 19(2): 42.

METCALFE, C.D. 1988. Induction of Micronuclei and nuclear abnormalities in the Erythrocytes of Mudminnows (Umbra limi) and Brown Bullheads (Ictalurus nebulosus) Bull. Environ. Contam. toxicol., 40: 489-495.

METCALFE, C.D. 1989. Tests for predicting carcinogenicity in fish. Aquatic Science, 1(1): 111-129.

METCALFE, C.D., G.C. BALCH, V.W.CAIRNS, J.D.FITZSIMONS and B.P. DUNN, 1990. Carcinogenic and Genotoxic activity of Extracts from contaminated sediments in western Lake Ontario. The science of the total environment, 94: 125-141.

MILLER, B.M. and M.NÜSSE, 1993. Analysis of micronuclei induced by 2-chlorobenzylidene malonitrile (cs) using fluoresence in situ hybridization with telomeric and centromeric DNA probes, and flow cytometry. Mutagenesis, 8(1): 35-42.

MINDGE, T., Y.I. YIZHEN and ZEN. ZHIYOUNG, 1987. Chlorothalonile inducted Micronucleus rate of EE1 erythrocytes. Human Yixueyuan xuebao (J.Human Med. Coll., 12: 153-154 (English summary).

MITROFANOV IUA., L.N.LESNIKOVA, A.Z.VOSKANIAN and V.V.OTRADNONA, 1991. Alternative and non - alternative process of the origin of chromosome aberrations. Radiobiologiia, 31(4): 585-592.

MOHN, G.R. and J.ELLENBERGER, 1976. Genetic effects of cyclophosphamide, Ifosfamide and Troforfamide. Mutation Res., 32: 331-360.

MORTIMER, R.K. and T.R.MANNEY, 1971. Mutation induction in yeast. In: Chemical mutagenes: principles and methods for their detection. Vol.1. Hollaender, A.(Ed.), Plenum press. 289-310.

MULLER, H.J. 1927. Artificial transmutation of the gene. Science, 66: 84-87.

NOMIYAMA, K., Y.SUGATA, A.YAMAMOTO and H.NOMIYAMA, 1975. Effects of dietary cadmium on rabbits. I. Early signs of cadmium intoxication. Toxicol. Appl. Pharmacol., 31: 4-12.

NIKINMAA, M. 1992. How does environmental pollution affect red cell function in fish?. Aquat. Toxicol., 22(4): 227-238.

NISHIMOTO, M., W.T.ROUBAL, J.E.STEIN and U.VARANASI, 1991. Oxidative DNA Damage in Tissues of English Sole (Parophrys vetulus) exposed to Nitrofuration. Chem. Biol. Interact., 80: 317-326.

NÜSSE, M., S.RECKNAGEL and W.BEISKER, 1992 a. Micronuclei inducted by 2-chlorobenzylidene malonitrile contain single chromosomes as demonstrated by the combined use of flow cytometry and immunofluorescent staining with anti-kinetochore antibodies. Mutagenesis, 7: 57-67.

OVEREND, W.G. and M.STACEY, 1947. Mechanism of the Feulgen nuclear reaction. Nature, 163: 538-540.

PARRY, J.M., D.J.TWEATS and A.J.Al-MOSSAWI, 1976. Monitoring the marine environment for mutagens. Nature, 264: 538-540.

PERRY, P. and S.WOLFF, 1974. New giemsa method for differential staining of sister chromatids. Nature, 251: 156-158.

PERRY P. and H.J.EVANS, 1975. Cytological detection of Mutagen-Carcinogen exposure by sister chromatid exchange. Nature, 258: 121-125.

PECHKURENKOV. V.L. 1991. The effect of the accident at chernobyl Atomic Electric power station in 1986 on the fish population of a cooling pond. Radiobiologiia, 31(5): 704-708.

PESCH, H.G. and C.E. PESCH, 1980. Neanthes arenaceodentata (Polychaeta: Annelida) a proposed cytogenetic model for marine genetic toxicology. Can.J.Fish. Aquat. Sci., 37: 1225-1228.

POWERS, D.A. 1989. Fish as model systems. Science 20: 352-358.

PROTIC, M.A. and ALEKSANDER-SABLJIC, 1989. Quantitative structure-activity relationships of acute toxicity of commercial chemicals of fathead minnors: effect of molecular size. Aquat. toxicol., 14: 47-64.

PUFFER, H.W., J.E.HOSE and V.M.LIGUORI, 1983. A method for evaluating pollutant teratogenicity and genotoxicity using freshwater or marine embryos. Fed. Proc. Fed. Am. Soc. Exp. Biol., 42: 351.

RAJU, M.B., M.S.KUSUMA and B.NEELAKANTAN, 1987. On some aspects of the maturation and spawning of the pearl spot Etroplus suratensis (Bloch) from the Kali Estuary, Karwar. MATSYA, 12-13: 39-44.

RANG, H.P. and M.M.DALE, 1987. Pharmacology. ELBS. 217-218, 368, 602-612.

REGAN, J.D., M.M.SIGEL, W.H.LEA, K.A.LEMES and A.R.BEASKY, 1968. Chromosomal alterations in marine fish cells in vitro. Can. J. Genetics and cytology, 10: 448.

REICHERT, W.L. J.E.STEIN, B.FRENCH, P.GOODWIN and U. VARANASI, 1992. Storage phosphor imaging technique for detection and quantitation of DNA adducts measured by the 32 p. Postlabeling assay. Carcinogenesis, 13(8): 1475-1479.

ROHRBORN, G. 1970. Chemical Mutagenesis in mammals and man, Vogel. f. and G.Rohrborn (Eds.), Springer-Verlag, Berlin, NY P.148.

SALAMONE, M., J.HEDDLE, E.STURAT and M.KATZ, 1980. Towards an improved Micronucleus test, studies on 3 model agents, mitomycin-C, cyclophosphamide, and dimethyle-benzanthacene. Mutation Res., 74: 347-356.

SCHMID, W., D.T.ARAKAKI, N.A. BRESLAV and J.C.CULBERTSON, 1971. Chemical Mutagenesis. The chinese hamster bone marrow as an in vivo test system.I. Cytogenetic results basic aspects of the methodology obtained with alkylating agents Humangenetik, 11: 103-119.

SCHMID, W. 1975. The Micronucleus test. Mutation Res., 3: 9-15.

SCHMID, W. 1976. The micronucleus Test for cytogenetic Analysis. In: Cehmical Mutagens, Principles and methods for their detection, Vol.4, Hollaender, A. (Ed.), Plenum Press. NY. 31-53.

SCHNITZ, A.R. and J.M., O. CONNOR, 1992. In vivo DNA/RNA adduction of 7, 12-dimethylbenz (a) anthracene (DMBA) and benzo(a) pyrene (Bap) in the liver of rainbow trout (Oncorhynchus mykiss). J. Environ. Pathol. Toxicol. oncol., 229-233.

* SCHROEDER, T.M. 1966. Cyto genetische and cytologische Befunde bei enzymopenischen penmyelo Pathien and Pancytopenien (amiliare Panmyctopathien Fanconi Glutathion reduktasemangel Anamie, Megaloblastare vitamin B folsauremangel Anamie) Humangenetik, 2: 287.

SCHROEDER, T.M. 1970. Investigations of bone marrow Smears. In: Chemical mutagens. Vogel. G. and G. Rohrborn (Eds.), Berlin Springer-Verlag.

SCOVASSI, A., R.WICKER and U.Bertazzoni, 1979. A phylogenetic study on vertebrate mitochondrial DNA polymerase, Eur. J. Biochem., 100: 491-496.

SIDDIIQUI, A. Q and S.M.NASEEM, 1979. The haematology of Rohu, Labeo rohita. J. Fish Biol., 14: 67-72.

SINDERMANN. C.J. 1980. The use of pathological effects of pollutants in marine environmental monitoring programmes. Rapp. R. -V.Reun. Cons. int. Explot. Mer., 179: 129-134.

SMIT, G.L. and J.HATTINGH, 1980. Haematological assessment of generally used freshwater fish blood anticoagulants. J. Fish Biol., 17: 337-341.

SMITH, I.R., V.E.VALLI, G.R.CRAIG, D.A.ROKOSH and H.F. FERGUSON, 1985. Ethylmethanesulfonate genotoxicity in Brachydanio rerio embryos. Can. Tech. Rep. Fish. Aquat. Sci., 1368: 345-350.

STACEY, N.H. and H.KAPPUS, 1982 b. Comparison of methods of assessment of metal-induced lipid peroxidation in isolated rat hepatocytes. J. Toxicol. Environ. Health., 9: 277-285.

STROMBERG, P.T., M.L.LANDOLT and R.M.KOCAN, 1981. Alterations in the frequency of sister chromatid exchanges in flat fish from puget sound. NOAA Technical Memorandum OMPA-10. Washington, USA.

SUGATT, R.H. 1978. Chromosome aberrations in eastern mud minnow U.pygmaea in vivo to Trenimon or river water. rep. M.D. and E. 78/3 of central Lab. Netherlands.

SUTOU, S. and CO-WORKERS, 1986. Sex difference in the micronucleus test. The colloborative study group for the Micronucleus test. Mutation Res., 172 (2): 151-163.

TATES, A.D., I. NEUTEBOOM, N. DE VOGEL AND L.DEN ENGELSE, (1983). The induction of chromosomol damage in rat hepatocytes and Lymphocytes. Time dependent changes of the clastogenic effects of diethyl nitrosamine, dimethylnitrosamine and ethyl methane sulfonate., Mutation Res., 107: 131 - 151.

TEACHER, A.E. 1970. Notes on Medical Jurisprudance and Toxicology. Current publishers, Calcutta. 171-186.

THOMAS, P. 1990. Molecular and Biochemical Responses of Fish to stressors and their potential use in Environmental monitoring. In: Biological indicators of stress in fish, Adams, S.M.,(Ed.) American Fisheries Symposium 8, Bethesda, Maryland 9-28.

TRIPATHI, S.D. 1989. Genetic difference in the morphology of erythrocytes of Labeo rohita (Ham) and Mrigal, Cirrhinus mrigala (Ham) as an adaptation to the environment. In: Fish Genetics in India, Das, P. and A.G.Ghingran, (Eds.) 245-249.

TYSTSUGIMA, V.G. 1972. In: Marine ecology, Polikarpov, G.G.(Ed.). AECtr 7299 Washington D.C. USA EC. P. 157.

VAN HUMMELEN, P., C.ZOLL, J.PAULUSSON, M.KIRSCH-VOLDERS AND A.JAYLET, 1989. The Micronucleus test in Xenopus: a new simple in vivo technique for detection of Mutagens in freshwater. Mutagensis, 4: 12-16.

VON LEDEBUR, M. and W.SCHMID, 1973. The micronucleus test. Methodological aspects, Mutation Res., 19: 109-117.

WALTON, D.G. A.B.ACTON and H.F.STICH, 1984. Comparision of DNA-repair synthesis, chromosome aberrations and induction of Micronuclei in cultered Human fibroblast, Chinese hamster ovary and central mudminnow (Umbra limi) cells exposed to chemical Mutagens. Mutation Res., 12(; 129-136.

WALTON, D.G., A.B.ACTON, H.F.STICH, 1988. Chromosome aberrations in cultured central mudminnow heart cells and chinese hamster ovary cells exposed to polycyclic aromatic hydrocarbons and sediment Extracts. Comp. Biochem. Physiol C. Comp. pharmacol. Toxicol., 89: 395-402.

WEI, Y.L. 1987. Preliminary studies on frequencies of sister chromatid differentiation (SCD) and exchanges (SCE) in renal cells of grass carp, Ctenopharyngodon idellus in vivo. Acta Hydrobiologica, 11(1): 29-33.

WILLIAMS, R.C. and C.D.METCALFE., 1992. Development of an in vivo hepatic micronucleus assay with rainbow trout. Aquat. Toxicol., 23: 103-202.

WRISBERG, M.N. and VAN DER GAAG, 1992. In vivo detection of genotoxicity in waste water from a wheat and rye straw paper pulp factory. Sci. Total Environ., 30(121): 95-108.

YAMAZAKI, F. 1983. Chromosomal changes in salmonids. Changes in chromosome number and morphology due to over ripening of eggs and irradiation. Can. Trans. Fish. Aquatic Sci. 4296: 1-22.

YAMAMOTO, K.I. and Y.KIKUCHI, 1980. A comparison of diameters of micronuclei induced by clastogens and by spindle poisons. Mutation Res., 71: 127-131.

YASUHIRA, S., H.MITANI, A. SHIMA, 1992. Enhancement of photorepair of ultraviolet-induced pyrimidine dimers by preillumination with flourescent light in the goldfish cell line. The relationship between survival and yield of phyrimidine dimers. Photochem. Photobiol., 55(1): 97-101.

YOUNES, M. and C.P.SIEGERS, 1984. Interrelation between lipid peroxidation and other hepatotoxic events. Biochem. Pharmacol., 33: 2001-2003.

ZHANG, Q., K.SUORSA-SUPER, L.R.CURTIS, 1992. Temperature-modulated aflatoxin B1 hepotic disposition and formation and Persistence of DNA adducts in rainbow trout. Toxicol . Appl. Phormacol. 113 (2): 253-259.

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* Not referred in Original.